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THE SURVIVAL OF SALMONELLA TYPHI IN SEWAGE OXIDATION PONDS

by

MICHAEL GRIMBLY McGARRY

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "THE SURVIVAL OF SALMONELLA TYPHI IN SEWAGE OXIDATION PONDS", submitted by Michael Grimbly McGarry in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Oxidation ponds are commonly used by small communities throughout Canada as a treatment process for their domestic wastes. S. typhi is the bacterium which causes typhoid fever, often the person who has contracted typhoid will remain a carrier of the bacteria following the disease's cure. As typhoid is a gastro-intestinal disease the excretions of the case or carrier will transport S. typhi to the sewage treatment system.

The objective of this investigation was to determine the longevity or survival of S. typhi in sewage oxidation ponds. These ponds may be divided into two categories, anaerobic and aerobic. The anaerobic ponds are used as primary ponds into which the raw sewage flows. The oxygen in this pond is used up by bacteria in breaking down organics. The aerobic pond follows the anaerobic in sequence. Because of longer detention periods and the fact that the larger particles have settled out and some decomposition has already occurred in the anaerobic, the oxygen is not completely utilized. Algae which grows in the aerobic pond is its main source of oxygen.

Direct inoculation of S. typhi into a pond in order to test the effluent for S. typhi and hence its longevity, was considered to be too dangerous. As a secondary investigation the death curves of the bacterium Serratia marcescens in sewage was observed. It was postulated that if S. marcescens and S. typhi had similar death curves, S. marcescens might be used to test ponds by inoculating it into the ponds and testing the effluent, thus indirectly obtaining an indication of the longevity of S. typhi.

The ponds were sampled through the use of large open ended pipes which were lowered into the ponds' waters. The samples were extracted by closing off the bottom end and raising the column by crane. After having

been placed under artificial lights in the laboratory of the Department of Microbiology of the University of Alberta the columns were fed with sterilized sewage thereby simulating the ponds under a wide range of loading rates. pH, alkalinities, residues and dissolved oxygen analyses were carried out during the investigation which showed that the columns simulated this wide range of pond conditions.

S. marcescens were inoculated into the sides of the columns and the death rates through the extraction of samples were observed. In like manner S. typhi was also inoculated and observed. Bismuth Sulphite agar was found to be entirely satisfactory in the isolation of S. typhi from sewage. The death rates of the two bacteria were not found to be similar. In view of this S. marcescens cannot be used in testing ponds for the longevity of the typhoid bacillus. The longevity of S. typhi was dependent upon the nutrient loading of the columns. Survival continued over periods of two to eleven days in columns which were being fed at rates of 50 to 6000 pounds of B.O.D. per acre of surface area per day respectively. In contrast to the aerobic columns, sewage addition was not carried out while S. typhi was present in the columns. It is postulated that S. typhi would have lasted for considerably longer periods of time had continual addition of sewage been maintained in the anaerobic columns.

The longevity of S. typhi in distilled and river water was also established which further verified the fact that S. typhi's survival in sewage is dependent upon available nutrients.

Batch tests of the survival of S. typhi in the ponds' waters were carried out over a range of temperatures. It was shown that S. typhi survived for longer periods of time at lower temperatures.

It was concluded that S. typhi can survive for extended periods

of time in anaerobic ponds and for only short periods in aerobic ponds. Hence the longevity of the typhoid bacterium in oxidation ponds was observed to be dependent upon the loading rates of the influent sewage.

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TABLE OF CONTENTS

		Page
Title Page		i
Approval Sheet		ii
Abstract		iii
Acknowledgements		vi
Table of Contents		vii
List of Tables		ix
List of Figures		xi
Abbreviations		xiii
CHAPTER I	THE OXIDATION POND	
I.1	A Review of the Oxidation Pond	1
I.2	Algae in the Aerobic Pond	3
I.3	Bacteria in the Aerobic Pond	4
I.4	Algal Bacterial Symbosis in the Aerobic Pond	5
I.5	The Ecology of the Anaerobic Pond	8
CHAPTER II	PUBLIC HEALTH ASPECTS OF THE OXIDATION POND	
II.1	The Present Oxidation Pond Controversy	10
II.2	Bacterial Pollution Indices	12
II.3	Incidence of Typhoid Fever	13
II.4	The Typhoid Bacillus	14
CHAPTER III	PROCEDURE AND METHODS	
III.1	Statement of the Problem	16
III.2	Proposed Techniques	17
III.3	Techniques Used	20
III.4	Limitations of the Investigation	22
CHAPTER IV	PREVIOUS LITERATURE PERTAINING TO THE SURVIVAL OF *S. TYPHI*	
IV.1	*S. typhi* in Nature	23
IV.2	*S. typhi* in Sewage Treatment Processes	24
IV.3	*S. typhi* in the Anaerobic Digester	24
IV.4	*S. typhi* in Oxidation Ponds	28

TABLE OF CONTENTS (continued)

		Page
CHAPTER V	METHODS AND EQUIPMENT	
V.1	The Sampling Columns	30
V.2	Internal Coating of the Columns	32
V.3	Lighting Fixtures	34
V.4	Nutrient Addition	35
V.5	Stirring Mechanism	37
V.6	Sampling of the Pond	37
V.7	Bacterial Enumeration Techniques	39
V.8	The Selection of Media for the Isolation of S. typhi	40
V.9	The Selection of Media for the Isolation of S. marcescens	45
CHAPTER VI	OBSERVATIONS	
VI.1	Chemical Analyses	47
VI.2	Observations of Tests Related to S. marcescens	60
VI.3	Observations of Tests Related to S. typhi	67
CHAPTER VII	DISCUSSION	
VII.1	Chemical Analyses	100
VII.2	Discussion of Tests Related to S. marcescens	102
VII.3	Discussion of Tests Related to S. typhi	104
CHAPTER VIII	CONCLUSIONS	107
CHAPTER IX	SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS	
IX.1	Summary of Conclusions	112
IX.2	Recommendations	113
Bibliography		

LIST OF TABLES

TABLE		Page
II.1	Incidence and Deaths Reported to the Department of Public Health of Alberta, Canada	13
II.2	Outbreaks of Foodborne and Waterborne Diseases Reported to the U.S.P.H.S. from 1952 to 1960, Typhoid Fever	14
VI.1	Anaerobic Pond, Chemical Analysis	50
VI.2	Aerobic Pond, Chemical Analysis	51
VI.3	Surface Dissolved Oxygen in Aerobic Columns	55
VI.4	Alkalinities in Anaerobic Columns	56
VI.5	Surface Alkalinities in Aerobic Columns	57
VI.6	Surface pH's in Aerobic Columns	58
VI.7	pH Levels in Anaerobic Columns	59
VI.8	Solids Content (Residues) mg/l in Anaerobic Columns	61
VI.9	Solids Content (Residues) mg/l in Aerobic Column	62
VI.10	*Serratia marcescens*, Growth Curve and O.D. Numbers	64
VI.11	Test Data for the Death Curves of *Serratia marcescens* in Anaerobic Columns	68,69
VI.12	Test Data for the Death Curves of *Serratia marcescens* in Anaerobic Columns, Averaged Values	70
VI.13	Test Data for the Death Curves of *Serratia marcescens* in Aerobic Column	71
VI.14	*Salmonella typhi*, Growth Curve and O.D. Numbers	78
VI.15	Preliminary Test Data for the Death Curves of *Salmonella typhi* in Distilled Water, Aerobic Column and River Water Samples	83
VI.16	Test Data for the Death Curves of *Salmonella typhi* in Anaerobic Column A	85
VI.17	Test Data for the Death Curves of *Salmonella typhi* in Anaerobic Column B	86
VI.18	Test Data for the Death Curves of *Salmonella typhi* in Anaerobic Column C	87

LIST OF TABLES (continued)

TABLE		Page
VI.19	Test Data for the Death Curves of Salmonella typhi in Anaeronic Column D	88
VI.20	Test Data for the Death Curves of Salmonella typhi in Aerobic Column	89
VI.21	Batch Test Data for the Death Curves of Salmonella typhi in Camrose Ponds	97

LIST OF FIGURES

FIGURE		Page
I.1	Schematic Diagram of the Bacterial - Algal Interaction in the Aerobic Pond	5
I.2	Cell Growth vs. Detention Period in an Oxidation Pond	7
III.1	Anaerobic Pond Sampling	18
III.2	Sample Columns	21
V.1	Anaerobic Sample Column	31
V.2	Inoculation Porthole	33
V.3	Sylvannia Daylight Fluorescent and Solar Radiation vs. Watts per 100 Angstrom Band	35
V.4	Settled Sewage Inoculated with S. typhi on Various Media	41
V.5	Settled Sewage Inoculated with S. typhi on Bismuth Sulphite Agar	43
V.6	Colony Counter, Refrigerator and Autoclaves	44
VI.1	Camrose Oxidation Ponds	49
VI.2	pH and Alkalinity of Anaerobic Pond	52
VI.3	Biochemical Oxygen Demand of Anaerobic Pond	53
VI.4	Alkalinities of Aerobic Pond	54
VI.5	pH of Aerobic Pond	54
VI.6	Temperature of Aerobic Pond	54
VI.7	Equipment Used in the Determination of Residues	63
VI.8	Growth and Optical Density Curves of S. marcescens	65
VI.9	Death Curves of S. marcescens in Anaerobic Column A	72
VI.10	Death Curves of S. marcescens in Anaerobic Column B	73
VI.11	Death Curves of S. marcescens in Anaerobic Column C	74
VI.12	Death Curves of S. marcescens in Anaerobic Column D	75
VI.13	Death Curves of S. marcescens in Anaerobic Columns, Averaged Values	76

LIST OF FIGURES (continued)

FIGURE		Page
VI.14	Death Curves of S. marcescens in Aerobic Column	77
VI.15	Number of S. typhi per ml. of Nutrient Broth vs. Time	79
VI.16	Growth Curve of S. typhi Optical Density Number vs. Time	80
VI.17	Number of S. typhi per ml. in Nutrient Broth vs. Optical Density Numbers	81
VI.18	Death Curves of S. typhi - Batch Tests	84
VI.19	Death Curves of S. typhi in Anaerobic Column A	90
VI.20	Death Curves of S. typhi in Anaerobic Column B	91
VI.21	Death Curves of S. typhi in Anaerobic Column C	92
VI.22	Death Curves of S. typhi in Anaerobic Column D	93
VI.23	Death Curves of S. typhi in Anaerobic Columns, Averaged Values	94
VI.24	Death Curves of S. typhi in Aerobic Column	95
VI.25	Death Curves of S. typhi in Anaerobic Pond - Batch Tests	98
VI.26	Death Curves of S. typhi in Aerobic Pond - Batch Tests	99

ABBREVIATIONS

Symbols are used in this report as abbreviated forms of the following:

O.D.	Optical Density Number
B.S.	Bismuth Sulphite agar
NAP	Nutrient Agar Plate
MBA	Methylene Blue Agar
O.R.P.	Oxidation Reduction Potential
mg.	Milligram
ml.	Millilitre
B.O.D.	Biochemical Oxygen Demand

CHAPTER I

THE OXIDATION POND

I.1 A Review of the Oxidation Pond

Throughout history mankind has encountered the problem of waste disposal. The largest public health hazard has been derived from the possibility of human contamination from fecal wastes. Due to the gastro-intestinal nature of many pathogenic viruses and bacteria, fecal excretion forms one path of the disease cycle. One recent method of waste disposal has come through the use of natural bio-degradation in sewage ponds. The sewage from a city is pumped into a series of ponds varying in area from one to several acres and in depth from four to fifteen feet. Through bacterial and protozoan action the sewage is then oxidized and 'cleaned'. It is then pumped either continuously or only during the summer months into nearby streams or reservoirs.

The oxidation pond's recent history and development have been reported by Van Eck (36) in his critical review of waste stabilization ponds. It was accidentally discovered in 1924 that adequate sewage treatment would be satisfactorily effected through an oxidation pond at Santa Rosa. By 1944 twenty-five pond installations were known to exist across California. By 1956 a total of 345 towns and cities in the United States were treating either raw or partially purified sewage by stabilization ponds. In Australia, as a result of studies by Parker (33), a rapid expansion in the use of oxidation ponds resulted in 750 acres of

pond being used at Melbourne in 1957, with the intention that 50 acres per year would be added. New Zealand has recently started to use the oxidation pond on a relatively small scale. Oxidation ponds in Holland have been described by Van Eck (36). By 1958 a total of 21 installations had been made in Sweden. In January of 1964 the Province of Alberta, Canada had 158 communities using sewage oxidation ponds, this represents 75% of the communities of Alberta being served by sewage treatment systems.

In 1961 a study of the operation of sewage ponds in the Province of Alberta was published by the Department of Public Health, Division of Sanitary Engineering (9). This study showed that there are many types of stabilization ponds used in the Province of Alberta which include the long detention pond, short detention pond, multi-cell (long or short detention) and a combination of short and long detention cells. The predominant type of pond used in Alberta is the long detention, single cell. These have detention periods of six months to a year or more and are drained twice a year (usually in the spring and fall). The short detention, single cell is used where a constant discharge from the pond is permissable. These ponds are anaerobic and have detention periods of up to ten days. The multi-cell, long detention ponds represent two or more ponds in series. Only five towns are presently operating on such a system. The multi-cell, short detention system is rare in Alberta, there being only one of the systems in existence consisting of three ponds in series. Their detention periods are up to ten days each. The combination of long and short detention ponds have shown favorable results in bio-degradation of organic wastes. Strong recommendations are being made by the Province for use of these in future installations.

I.2 Algae in the Aerobic Pond

The biological life in the sewage oxidation pond is highly complex. In order to gain some knowledge of the processes within the pond, an understanding of the biota is required.

Algae is common in the aerobic pond and performs a very definite and useful function. The most common genera found in aerobic stabilization ponds are Chlorella, Scenedesmus, Euglena and Chlamydomonas. Their relative populations vary with the particular detention period of the pond and type of substrate (18). Algae contain a chloroplast including chlorophyl 'a'. This gives them the property of photosynthesis (34) and the ability to perform the following basic and general reaction:

$$CO_2 + H_2O \xrightarrow{\text{alga+light}} CH_2O + O_2 \qquad (1)$$

The oxygen thus produced is utilized by bacteria in the aerobic stabilization pond to oxidize the organic material. "The amount of plant material produced is largely a function of the amount of CO_2 and light energy available"(31). William J. Oswald et al (31) have reported on the effects of light energy on algae in ponds. They conclude that too much light is harmful to the single cell but when a mutual shading effect is available from other algal cells the culture can grow effectively and abundantly at very high light intensities. A continuous culture of Euglena was studied for concentration of cells and chlorophyl content over various lighting schedules at 800 foot candles. These schedules were continuous lighting, 16 hours on and 8 hours off, 40 seconds on and 20 seconds off. The culture with the 24 hour cycle produced twice as much plant growth or yield as the one minute cycle

and 1.4 times the yield of the culture with the continuous lighting.

There was a positive correlation found between the yield and the chlorophyl content of the cell. There has been a continued and enlightening program of research on algae in the sanitary engineering laboratories at the University of California, Berkely. With reference to (18) studies have included algal-bacterial symbiosis in sewage oxidation ponds, studies of photo-synthetic oxygenation, waste water reclamation through the production of algae and methane fermentation in sewage ponds.

I.3 Bacteria in the Aerobic Pond

The general cycle of symbiosis is completed through the ability of bacteria in performing the following reaction:

$$CH_2O + O_2 \xrightarrow{\text{bacteria}} CO_2 + H_2O \qquad (2)$$

The following cycle is carried out through the interaction of the above simplified reactions(1), and(2) (FIGURE I.1). The sewage influent into the oxidation pond contains nutrient materials including phosphorous, magnesium, carbon, nitrogen and sulphur.

The bacteria are capable of decomposing the nutrients, originally in complex form, releasing carbon dioxide ammonia and other products. Algae require ammonia as their main source of food. As a product of metabolism, algae release oxygen required by the aerobic bacteria to synthesize organic wastes. FIGURE I.1 indicates the relative amounts of nutrient materials by the width of arrows.

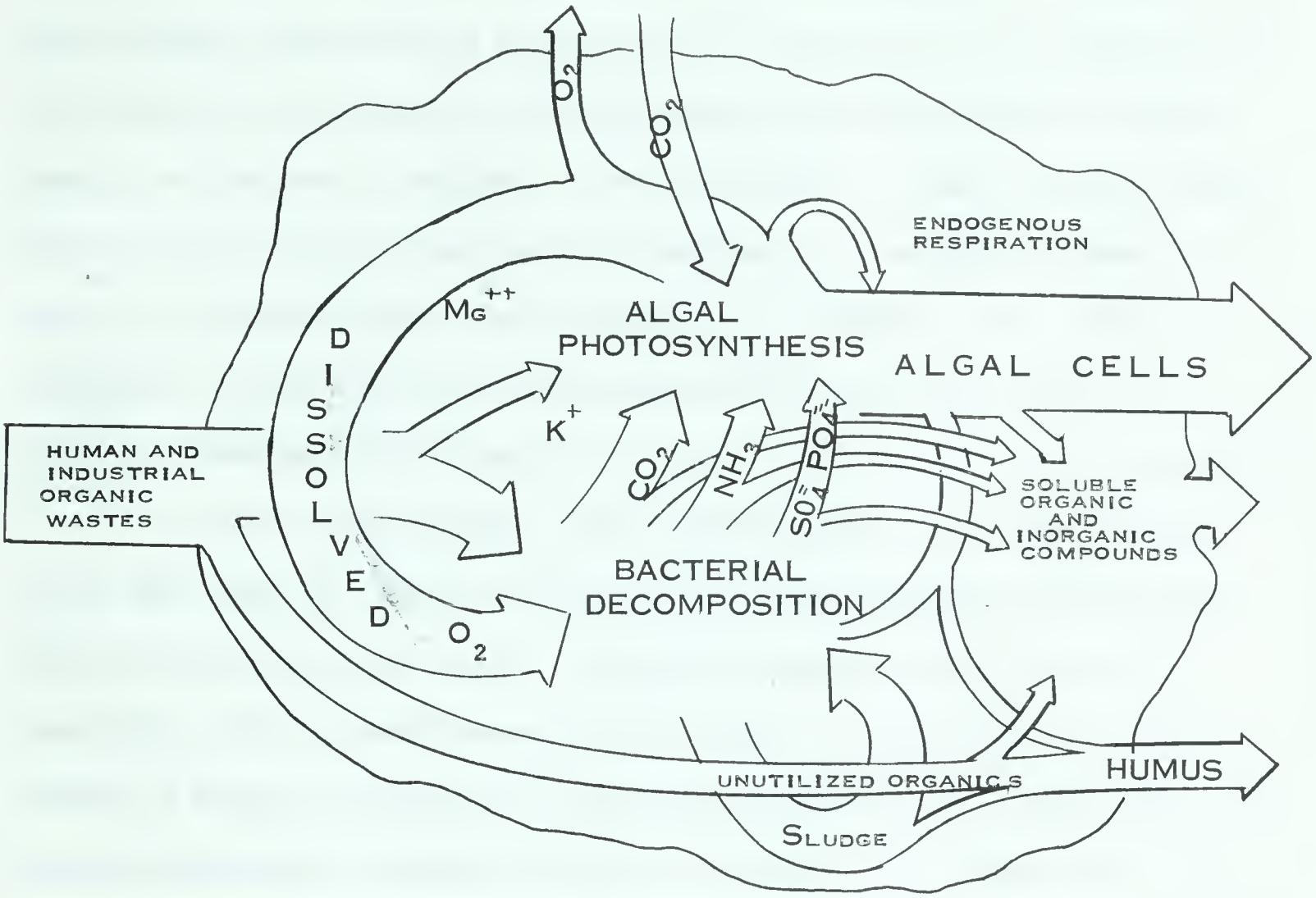


FIGURE I.1 Schematic Diagram of the Bacterial-Algal Interaction in the aerobic pond. Reproduced from (19).

I.4 Algal-Bacterial Symbiosis in the Aerobic Pond

It has been observed (31) that due to the action of algae growth in raising the pH of the pond during the day, bacterial action is considerably reduced. With the reduction of algal photosynthesis at night the bacteria quickly regain their metabolic activity under more favorable pH conditions. As the influent sewage into the primary ponds have little to no algal cells the inoculation of algae into the

system comes through natural processes. Simultaneous interaction between algae and bacteria seldom takes place, unless so induced. Induction may come through recirculation as suggested by Oswald et al (31) which would introduce new algal cells to the influent creating an immediate oxygen supply for the use of bacteria. The common use of long detention period ponds of 20 to 35 days may cause the effluent of the pond to increase in BOD, due to the dead algal cells which are not readily broken down by bacteria. In the long detention ponds the yield of algal cells is reduced considerably (30) with a consequent reduction of oxygen production.

Due to the effective light intensities seldom being reached below the level of 3 feet in the pond (32) more effective ponds may be designed with shallower depths. Designers are presently hesitant to construct shallow ponds because of the danger of weeds and the ensuing growth of mosquitoes capable of spreading encephalitis. Paved ponds may become economically feasible in the near future. By a reduction in the depth, the pond acquires a shorter detention period having a larger effective surface area per volume.

Studies (31) were carried out on the yield of Euglena gracilis, Scenedesmus obliquus, Chlorella pyrenoidosa and the common sewage bacteria with the view of comparing the yield of cells to the detention period. The detention period is

$$R = V/v \tag{3}$$

where 'R' is the detention period in days, 'V' is the volume of the pond under consideration and 'v' is the influent volume per day. A plot of the results by Oswald et al is given, FIGURE I.2.

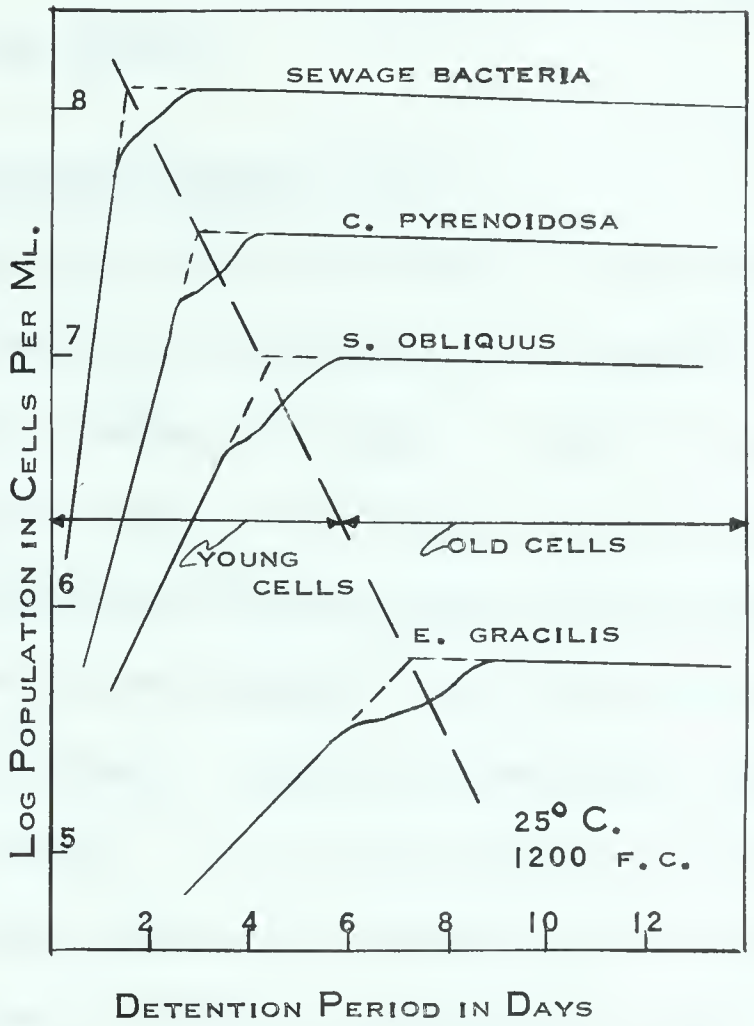


FIGURE I.2

Cell Growth vs. Detention Period in an Oxidation Pond
Reproduced from (31)

It may be seen that the maximum yield of algae comes at detention periods which are much longer than those at the maximum yield of bacteria. Due to the more effective cells being those on the log-growth phase it is desirous to have young cells in the ponds at all times. In view of this, the bacterial growth is governed to an extent by the amount of oxygen which the algae produces. A satisfactory detention period is one which may keep the algal cells at their maximum activity, the bacterial cells being able to accomodate the oxygen as

rapidly as it is produced by the algal cells. Ultimately the oxidation pond should be designed for the production of viable algal cells after weeds are taken into account.

I.5 The Ecology of the Anaerobic Pond

Throughout the study of the ponds it was noted that the dissolved oxygen content of the primary short detention ponds remained zero at all depths including the surface. There has been some controversy over the nomenclature of the ponds. In view of the fact that there are no obligate aerobes and no free oxygen in the primary short detention ponds they shall be called 'anaerobic' and the representative columns 'anaerobic' sample columns. Likewise the secondary long detention ponds shall be termed 'aerobic'. It is postulated that although there is a wide temperature range between the sludge digester and the anaerobic pond, their ecologies are similar recognising the fact that psychrophilic organisms inhabit the pond and thermophilic the digester.

Langley et al (27) suggest that the organisms in the anaerobic digesters may be set into two classifications. (a) The methane producers being mostly strict anaerobes and (b) the acid formers, being mostly facultative, remain essentially in the same numbers whether in the aerobic surroundings or the anaerobic. In the anaerobic state the biodegradation of organics is exothermic whereas in the aerobic degradation excess energy is not released in so great proportions. Essentially, part of the organics is used as materials for the synthesis of protoplasm and part oxidized for use as energy. Thus the bacterium must oxidize more material in the anaerobic state for the same amount of energy it might gain in the aerobic.

It has too often been stated that there can be complete stabilization of all organic matter in the anaerobic lagoon. This is not so. Oxidation is basically a transfer of hydrogen, the hydrogen acceptor in the aerobic pond is oxygen leaving a net yield of energy to the bacterium. In the anaerobic case the hydrogen is donated to organic material at a lower energy level than that of the substrate which is being oxidized. Thus, due to the continual receipt of hydrogen, the materials at the lower levels are never oxidized as their function remains as hydrogen acceptors. It is for this reason that the bacterial populations in the aerobic ponds do not attain levels in proportion with the B.O.D. of the surroundings. The bacteria exist in a state of starvation which intensifies with longer retention periods.

CHAPTER II

PUBLIC HEALTH ASPECTS OF THE OXIDATION POND

II.1 The Present Oxidation Pond Controversy

The majority of the ponds of Alberta are unguarded and unfenced, some discharge their effluent into nearby rivers and streams on a continuing basis. If pathogenic bacteria and viruses are able to remain viable or multiply through the process of bio-degradation in the ponds there is a great danger to the health of the neighboring communities. Percolation through the soil is also regarded as a health hazard.

In recent months a controversy has been stirred up through the medium of the Financial Post over the disease spreading potential of oxidation ponds. In an article (25), McAnulty states "evidence accumulates to indicate that lagoons (there are at present over 2000 in North America) are sources of communicable diseases such as zoonoses through rural and domestic cycles, involving arthropods, rodents, fowl, reptiles, and amphibia, man and animals (domestic and wild) - man and beast essentially being effected tangentially . . . . Lagooning of raw wastes constitutes a health hazard and violates one of the concepts of modern waste treatment. Since this primitive practice provides an excellent opportunity for the transmission and the spread of communicable diseases, there exists at present a potential unchecked plague". He further states that the most important vector-bourne disease in North America is the mosquito-borne viral encephalitides which causes the "production of alarmingly serious

illness, permanent impairment and even death among humans" (25). This is the greatest health hazard created by the pond and although in the original publication he does not mention typhoid, Danude (8) quotes McAnulty as saying towns using this cheap method of sewage disposal are putting themselves in line for a potential unchecked plague of communicable diseases, such as "hepatitis, encephalitis (inflammation of the brain) Salmanella (source of gastro intestinal infection, typhoid fever, acute diarrhea)". More recently in the Financial Post of January 30th, 1965 (11), an article reported that "scores of newspapers printed summaries of the Financial Post report and many wrote asking whether their townships' lagoons were in fact a hidden menace. An Ontario community suspended work on a new lagoon until it got assurances from an international firm of sanitary engineers that in this particular case there should be no danger to health" (11). The Saskatchewan Department of Health has issued a statement intending to reassure the 250 communities of the province which are using lagoons that the health hazards are negligible. Dr. W. Harding le Riche (21) states that "nobody can say he is sure that sewage lagoons are perfectly satisfactory. They may be, and certainly I know of no evidence to create general public alarm, but then again, they may be only partially satisfactory". Mr. M. Walkinshaw (37) states that "I know of no public menace from well made, well sited and well maintained lagoons. In fact, one can think of instances where a lagoon would be a better solution to the sewage problem than an expensive mechanical treatment plant".

In the light of nebulous statements such as these and the continuing controversy, the Department of Public Health of the Province

of Alberta decided to incorporate the public health aspect of stabilization ponds into its "Study of the Operation of Sewage Ponds in the Province of Alberta" (9).

II.2 Bacterial Pollution Indices

The bacteriological quality of water is tested through the enumeration of coliform bacteria. "Standard Methods for the Examination of Water and Waste Water" as published by the A.P.H.A. and A.W.W.A. and W.P.C.F. (the American Public Health Association, American Water Works Association, and Water Pollution Control Federation Journal) (1) recommends the use of the coliform group of bacteria to indicate pollution of water with sewage and waste. Experience has established the significance of coliform group densities as criteria of the degree of pollution shown by the bacterialogical results, and thus of the sanitary quality of the sample under examination. The coliform group includes all bacteria which are aerobic obligate and facultative aerobic, Gram-negative bacillic and form gas through fermentation of lactose within 48 hours at 35°C. S. typhi is also a gastro-enteric bacillus and qualifies as a coliform in all respects other than its not being able to ferment lactose with gas production within 48 hours at 35°C. "The isolation of pathogenic bacteria or other micro-organisms from water and sewage cannot be recommended as a routine practice, since the techniques available at the present are tedious and complicated, and require so long a time that the results when obtained are no longer of significance and may be confusing in a particular study of pollution" (1). There is however some confusion as to the reliability of using the coliform bacteria. The World Health Organization recommends counts of faecal streptococci and Clostridium

welchii and bacterio-phages as well as the coliform organisms (40). The use of Streptococcus faecalis is made commonly in England in the bacteriological examination of water.

Although the coliform group has been generally accepted to indicate bacterialogical contamination of waters, the comparative longevity of the coliform and typhoid bacilli in oxidation pond waters has not been established to date. In view of this,coliform bacteria to indicate the rate of death of typhoid in ponds could not be used.

II.3 Incidence of Typhoid Fever

Although deaths resulting from typhoid in the past 10 years have reduced considerably, outbreaks and cases still continue to some degree. The following table indicates the number of cases of typhoid caused by Salmonella in the United States and the Province of Alberta as reported to the Departments of Public Health:

TABLE II.1

INCIDENCE AND DEATHS REPORTED TO THE DEPARTMENT OF PUBLIC HEALTH OF ALBERTA, CANADA

Year	Cases	Deaths
1964	4	0
1963	2	0
1962	8	0
1961	16	0
1960	3	0
1959	13	0
1958	6	0
1957	16	2
1956	22	1
1955	8	2
1954	5	1
1953	9	1
1952	6	2
1951	37	0

TABLE II.2

OUTBREAKS OF FOODBORNE AND WATERBORNE DISEASES REPORTED TO THE U.S.P.H.S. FROM 1952 TO 1960, TYPHOID FEVER (5)

Year	Outbreaks	Cases
1952	11	156
1953	12	75
1954	16	92
1955	5	36
1956	7	52
1957	4	70
1958	1	30
1959	5	43
1960	4	39

II.4 The Typhoid Bacillus

Typhoid fever is caused by the bacteria *Salmonella typhi*. *Salmonella typhi* is a rod-shaped bacterium (bacillus), which has the characteristics of being flagellated, Gram-negative and produces characteristic biochemical reactions in culture. They are susceptible to heat, cold, radiation and disinfectants (5).

The genus *Salmonella* of which *typhi* is one species can be killed by autoclaving at 15 lbs per sq. inch and 121°C for fifteen minutes. They are killed by the usual practices of pasteurization. The genus *Salmonella* has so far revealed more than 800 types of bacilli amongst which the species *S. typhimurium* is the most common in man. Salmonellosis is most common in poultry, livestock and rodents and may also be found in other wild-life, birds, animals, reptiles and other anthropods.

As the usual habitat of the genus is in the gastrointestinal tract, the path of transmission is the fecal-oral route. Humans may become infected through the contamination of human food, animal food, water supplies, sewerage systems and soils by feces.

It has been found that "Refrigeration . . . does not destroy Salmonellae but merely prevents their multiplication. . . In Canada, during the period 1951 to 1955, the incidence of Salmonellosis showed a marked increase during the month of June, reaching a peak in August. The incidence remains relatively high in September and October but falls off during the winter months to approximately 50% of the level experienced during the summer months. Similarly in British Columbia, the incidence in the six warmer months, April to September, was almost twice that during the six colder months, October to March" (5).

With Salmonellosis the infection causes the infected to carry the bacilli in the intestinal tract for a short time after the illness. Temporary carriers are relatively common. The state of being a carrier lasts usually for periods of two to three weeks. Chronic carriers are able to excrete Salmonellae for periods up to a year after the infection. The carrier state resulting from S. typhi infection is quite a different matter. 3% of the patients of typhoid excrete the organism indefinitely (5). If there is such a carrier in a community using oxidation ponds as its waste treatment process, there is a continual influx of S. typhi into the pond endangering perhaps health in the neighbouring district. It is for this reason that a thorough investigation into the effectiveness of the ponds in killing the organism was carried out.

CHAPTER III

PROCEDURE AND METHODS

III.1 Statement of Problem

Essentially the problem was to discover whether or not the typhoid bacillus was capable of remaining viable through the oxidation pond stabilization treatment processes.

Ultimately this would require the death rate curves of the bacillus in all physical, chemical, and biological conditions found throughout the entire process. This was not possible due to the amount of time and funds available. In view of this, the death curves of the bacillus S. typhi were to be obtained in aerobic and anaerobic ponds under a reasonable variety of conditions.

As an extension of the investigation an indirect method of testing ponds for the longevity of S. typhi was approached. Serratia marcescens, a bacillus which pigments red on nutrient agar plates, has a similar metabolic path to S. typhi. However it offers the facility of not being pathogenic. It was advanced that a comparison of death curves under similar conditions between S. typhi and S. marcescens be made with the view of uncovering an indirect testing procedure without the health hazard. If the death curves of the two species were similar, S. marcescens might be used in future to test pond waters for the longevity of the typhoid bacillus through its inoculation into the pond.

III.2 Proposed Techniques

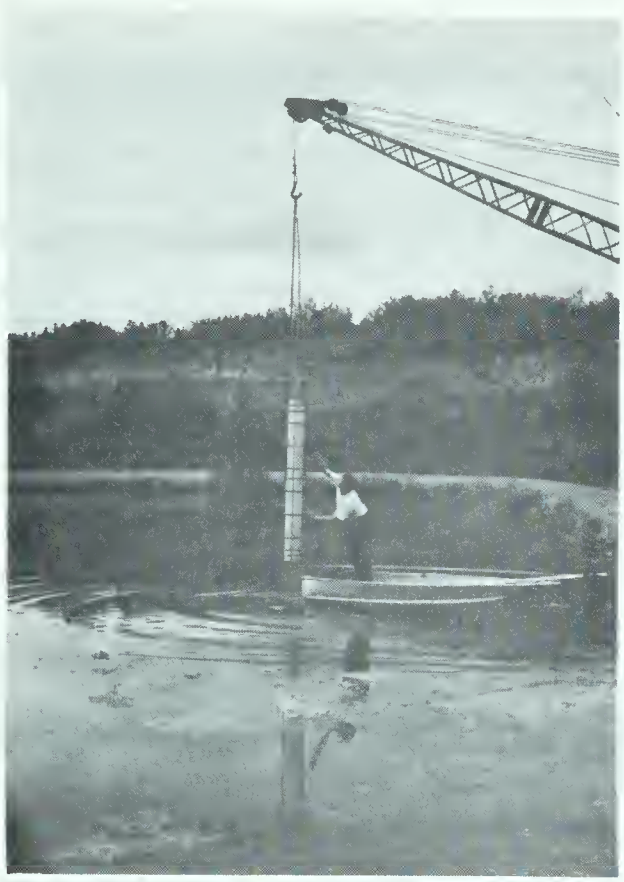
Three approaches to the problem could have been made.

(1) A direct inoculation of large quantities of S. typhi bacteria could have been made into an actual oxidation pond. Through the bacterialogical analyses of the effluent and of several samples taken from various locations in the pond, a death curve of S. typhi could have been obtained. The health hazard to the neighbouring district was considered too great by the Department of Public Health of Alberta, to carry out this scheme. The flow pattern varies considerably from pond to pond resulting in further complications.

(2) Large oxidation ponds of a similar shape as found in the field could have been set up in the laboratory and lighted by artifical lights and fed through the continual influent of raw sewage. Inoculations could have been made into the influent and death curves obtained through a similar procedure as (1). Neither a source of continual raw sewage, nor the equipment was available.

(3) A laboratory study using columns, provided the possibility of setting up a column or pipe in the Micro-biology laboratory for future tests, as shown in FIGURE III.1. The columns could be fed with nutrient in the form of raw sewage while outdoor conditions such as light could be applied. Testing of the columns at various heights might then be effected through the inoculation and extraction of samples through the sides of the column.

Nutrient addition would be made through small taps above the aerobic columns. The nutrient would be fed by a gravity system. Lighting fixtures above the aerobic columns would be maintained at various heights above the surface for various light intensities. In order to simulate outdoor conditions, stirring of the sample column would be effected through



[A] Lowering the Column by Pulley

[B] Preparation of the Column

[C] Loading the Columns into the Truck

[D] Tightening the Tension Bar Nuts to Seal off the Base Plate

Anaerobic Pond Sampling

FIGURE III. 1

an iron bar coated with plastic located in the center of each column driven by a motor and gear system above the columns themselves. Nutrient addition to the anaerobic columns would entail a daily portion of raw sewage sludge fed to each column.

The death rates of Serratia marcescens and Salmonella typhi could be obtained by an original inoculation through holes in the sides of the columns (port holes) and thence an enumeration of the number of remaining viable bacteria through extractions.

The third procedure was considered the most feasible by the author. The bacteria were to be inoculated in proportions of approximately 10^5 per ml. and then through the use of differential media on millipore filters and field monitors, their death rates observed. It was hoped that a wide variety of conditions and a great number of death curves would be acquired through the varying height of lights over the aerobic columns thus changing the constituents of the sample sewage. The nutrient addition being variable would also enable more death curves to be obtained under further chemical and biological conditions. As lighting and stirring have minimal effects in the anaerobic pond, the only variable between the columns would be that of nutrient addition.

In order to estimate the number of bacteria going into the columns, plots of optical density numbers versus number of bacteria were to be made. The Serratia marcescens and Salmonella typhi cultures were to be grown at 37°C over a period of 24 hours and through the use of culturing techniques on agar plates, the number of bacteria at any given time might be estimated. The spectrophotometer was to be used in estimating the optical density number of the culture so that plots of optical density versus the number of bacteria per ml. might be obtained.

III.3 Techniques Used

It was found during the actual testing procedure that certain changes in the proposed techniques were necessary. Following the sampling of the ponds as described by FIGURE III.1 the columns were taken back to the Department of Microbiology of the University of Alberta and located side by side under the dexion frame and lighting fixtures as shown by FIGURE III.2. The stirring rods were located inside the aerobic columns and turned on for continuous mixing. Nutrient for the aerobic column was obtained from the Main Sewage Treatment Plant of the City of Edmonton, Alberta in the form of settled sewage. This was autoclaved and placed in large jars above the columns to be emitted through small taps into the sample columns as shown in FIGURE III.2. The lights were turned on for continuous lighting. under these conditions it was assumed that the growth of algae would be maintained in similar proportions to the ponds. However the continued depression of the original dissolved oxygen content showed that this was not happening. To raise the oxygen content air was then pumped through a glass tube extending to the bottom of the columns according to the procedure of (31). The bubbling caused a mixture of the sample waters obviating the need for a mechanical stirring device.

It was observed that the lowest position of the lighting fixtures was 11" above the surface of the water. At this height, only 600 foot candles of light were received by the surface of the water. This is comparable to the light intensities of a very cloudy day or dusk. Under these conditions the algae grew only very slowly but did maintain the dissolved oxygen slightly above the saturation level as indicated on TABLE VI.3.

Great difficulties were encountered in the addition of nutrient to the aerobic columns. Complete blockage of the taps necessitated the

[A] Sample columns, general view

[B] Aerobic Sample Columns, detail

Sample Columns

FIGURE III. 2

filtration and coagulation through the filtration and sterilization of the raw settled sewage. The most efficient procedure was found to be the autoclaving of the settled sewage as it came from the treatment plant, then filtration through a number 2 filter* and a further autoclaving before storage and use. Further blockage by algae necessitated that the whole addition apparatus be autoclaved. Even with these precautions only an approximately constant nutrient addition was maintained through daily adjustment. It was observed that a constant flow pump and control device as used in (31) would be necessary for constant nutrient addition.

III.4 Limitations of the Investigation

The public health hazard of the inoculation of S. typhi directly into the ponds was avoided through the use of sample columns. The columns offered a most effective simulation of outdoor conditions as shown by FIGURE III.2. . Although a better distribution of sampling throughout the ponds would have given a variety of conditions in the columns this was effected by varying the nutrient addition. In this way a variety of ponds under different loading conditions were represented.

Many strains of S. typhi have been discovered, all having very similar metabolic pathways. Only one strain was studied (A40), being representative of the species Salmonella typhi. It was originally assumed that Serratia marcescens would have a similar death curve to that of S. typhi. Following tests on Serratia marcescens this postulation was shown to be incorrect as shown by FIGURES VI.9 to 14. However, this did not in way effect the tests and resulting death curves of Salmonella typhi.

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* Whatman Number 2 Filter - W. and R. Balston Ltd.

CHAPTER IV

PREVIOUS LITERATURE PERTAINING TO THE SURVIVAL OF *SALMONELLA TYPHI*

IV.1 *S. typhi* in Nature

P.J. Beard presented a paper entitled 'The Survival of Typhoid in Nature' to the American Water Works Association in 1938 (3) in which he covered much of the previous work on the subject of *S. typhi* survival in nature. One of the earliest workers was E.O. Jordan (1904) who successfully demonstrated to the City of Chicago that the typhoid bacillus was unable to withstand the voyage down the river to St. Louis (23). Studies were made on the survival of *S. typhi* in polluted sea waters (2) in 1935 by P.J. Beard. In 1917, Melick (28) found that the typhoid bacillus survived in feces mixed with soil for periods as long as 25 and 41 days. Galvagne (14) demonstrated that infected feces on the surface of soil would remain so for ten days and the underlaying soil layers would be infected by *S. typhi* for periods up to 40 days.

Beard, Carlson and Chambers (4) studied the survival of the typhoid bacillus in adobe, adobe and peat mixture, and peat under varying weather conditions. Stating that the longest period of survival was 50 days in the loam under wet conditions, they also give a minimum survival of 2 to 7 days in sand depending on humidity and temperature. The concluded with the statement that "other conditions being equal, the lower the temperature the longer the survival . . . another possible factor that we have not been able to evaluate is the effect of other soil organisms". As will be shown the competition for food resulting from the presence of

other organisms is the primary factor in the survival of S. typhi.

IV.2 S. typhi in Sewage Treatment Processes

Bruns and Sierp (6), Wilson Blair and Ruchhoft (38) and Heukelekian and Schulhoff (22) studied the death rates of S. typhi in the activated sludge process and all observed an above 90% reduction within six hours detention.

Green and Beard (20) performed a series of experiments on the "Survival of E. typhi in Sewage Treatment Plant Processes". Using wide mouth jars and raw sewage inoculated with typhoid, they were able to follow the death rate. S. typhi lasted in the jars for 33 days. Through a simulated septic tank, successful isolations in 1 ml. were made in the supernatant for 24 days and in 0.1 ml. portions of the mixed sewage for 27 days. Activated sludge treatment plants were simulated in the laboratory with results comparable to those previously mentioned (6), (38) & (22) although actual death curves were established in this instance.

Studies on trickling filters were made by the addition of S. typhi to the influent being filtered through a simulated trickling filter at rates of 2.8, 6.6 and 12 mgd. Reductions of 99.9%, 96-99% and 95% were observed respectively in the counts of the typhoid bacillus. In the conclusion of his paper, Beard (20) states, "In all of our experiments cited we observed that the vast majority of the pathogens are quickly eliminated . . . We are not concerned with the percentage that die, but with the small percentage that survive. Our objective is not to render water less dangerous but to render it safe."

IV.3 S. typhi in the Anaerobic Digester

R.E. McKinney et al (26) studied the survival of Escherichia coli,

Aerobacter aerogenes, S. schottuelleri, Shigella dysenteriae and S. typhi in anaerobic digestors. Following the choice of Bismuth Sulphite agar for his selective media for S. typhi he went on to test the death rates of S. typhi inoculated into simulated digesters. The digesters were six litre units and feeding was controlled by the addition of sludge from a local municipal sewage treatment plant effecting detention periods of 6 and 20 days. As the sewage from the municipal treatment plant did not offer the correct pathogenic organisms in any workable quantity, it was decided to inoculate the digester units with the suspended organism daily. Counts were made on the sludge mix before and after inoculations. An average of 44.7×10^4 S. typhi per ml. was added by this daily inoculation technique in both digesters. The reduction of numbers was noted to be greater in the twenty day digester than in the six after a twenty-four hour period had elapsed. Reductions to 3.4×10^4 per ml. (92.4% reduction) in the 20-day digester and to 7.7×10^4 per ml. (84.0%) in the six day digester were observed.

It was concluded that the more rapid die-off was due to the relative shortage of food in the digester effected by the longer detention period.

Langley, McKinney and Campbell continued their work on the "Survival of Salmonella typhosa during anaerobic digestion" with a second paper entitled "II. The Mechanism of Survival" (27). It was postulated that the oxidation - reduction potential (O.R.P.), available nutrients, the nature of the gases present, the presence of antibiotic substances, the presence of bacterio - phages and the competition of other bacterial species for food acted as the main influences in the survival of S. typhi.

Following the growth of S. typhi in nutrient broth for 48

hours at 37°C, the O.R.P. of the suspension was observed to be -154 mv (corrected to the hydrogen scale). This indicated that S. typhi could grow well at the O.R.P. levels of the digesters and that O.R.P. was not a serious factor in the death of S. typhi in the digesters.

Growth studies were made of S. typhi, Aerobacter aerogenes and E. coli in individual sterilized sewage sludge samplesobtained from a local municipal sewage treatment plant. Being inoculated into the 20 day digester as previously described, all were able to utilize the available nutrients in the sludge and maintain levels of 10^8 bacteria per ml. for a period of 24 days. Following this, inoculations of 10^3 bacteria per ml. were made into individual digesters of sterilized raw buffered sludge of A. aerogenes, E. coli, and Pseudomonas fluorescens. To each of these 10^3 S. typhi per ml. was added. The ensuing growth curves showed that there was a definite competition for food between S. typhi and the other bacteria, S. typhi never being able to reach or maintain the same population numbers as its complementing bacterial species.

The effect of the anaerobic gaseous atmosphere was studied through sterilized sewage sludge being inoculated in a similar manner but this time it was kept under a nitrogerous atmosphere, a methane atmosphere and an aerobic atmosphere. It was postulated that methane would act as an enzyme inhibitor. However, the results did not support this postulation.

To study the effect of bacteriophages and antibiotics present in normal sewage on S. typhi, two samples of the supernatant were passed through a biological filter, one of which was sterilized by heat. The resulting inoculation of S. typhi in both samples indicated that death was possibly due to the phages, antibiotics or simply the lack of the

essential food in the supernatant liquid. In this light, a similar study was carried out with the addition of nutrient broth to the supernatant. Growth occured and maintained population levels similar to those observed in pure nutrient broth. Langley et al concluded that the previous death rate observed in the broth deficient samples was due solely to the lack of food in the supernatant and not to the antibiotics nor to the phages. It is noted at this point that this test was sufficient to exclude the possibility of phage interference but not the antibiotic interference.

As the foregoing studies concluded that the reason for the death of S. typhi in digesters was the competition of other bacterial species for food and that this food was not available in sufficient quantity in the supernatant liquid, a preparation of filtered, dehydrated and pulverized sludge solids was made. This was resuspended in distilled water and inoculated with the same organisms in a similar manner as previously described. The resulting growth patterns showed that S. typhi could not predominate in the presence of other test organisms even when the other organisms were inoculated in small concentrations after S. typhi had reached its equilibrium concentration in the culture. This showed that the other organisms were able to compete successfully for the food.

To determine the essential metabolite, samples of dried sludge were extracted with petroleum ether. Through the inoculation of S. typhi into the ether soluble and ether insoluble portions, the ensuing growth curves indicated that the essential metabolite was in the ether insoluble fraction and hence was not in the fat component of sludge.

The resuspended sludge solids in distilled water were acid and

base hydrolized and the resulting samples were given population numbers of 3.4×10^3 S. typhi per ml. The growth curves of the base hydrolysates increased to population levels of 10^7 per ml. while those of the acid hydrolysates showed rapid death. Through a chromatographic analysis of the neutral soluble fractions of the acid and base hydrolysates, it was concluded that the essential metabolite was not a carbohydrate. The comparison of the am ino acids of both factions pointed to tryptophan as being the essential metabolite.

To further check the above analyses, tryptophan was added to acid hydrolyzed nutrient broth. The growth curve of S. typhi in this medium was compared to those obtained when plain nutrient broth, acid hydrolyzed nutrient broth fortified with tyrosine and acid hydrolyzed nutrient broth fortified with both tryptophpan and tyrosine were inoculated with S. typhi. The results showed that "there was no doubt, that the amino acid tryptophan was the metabolite in sewage sludge essential to the growth and survival of S. typhosa" (27). Further studies of digesters fortified with tryptophan added strength to this conclusion.

IV.4 S. typhi in the Oxidation Pond

In the publication "Removal of Pathogenic Micro-organisms by Sewage Treatment Processes" (24) Kabler makes reference to Fitzgerald and Rohlich (12) in stating that "the E. Coli have generally been reduced from several hundred thousand to less than 100 per ml., and one case has been reported where typhoid bacteria were reduced from 41 per ml. to negative results." In this publication (12) several references are made to tests carried out across the United States, Australia, Denmark, Sweden and

Germany, where the coliform counts were sufficiently reduced to less than 100 per ml. of effluent from aerobic oxidation ponds.

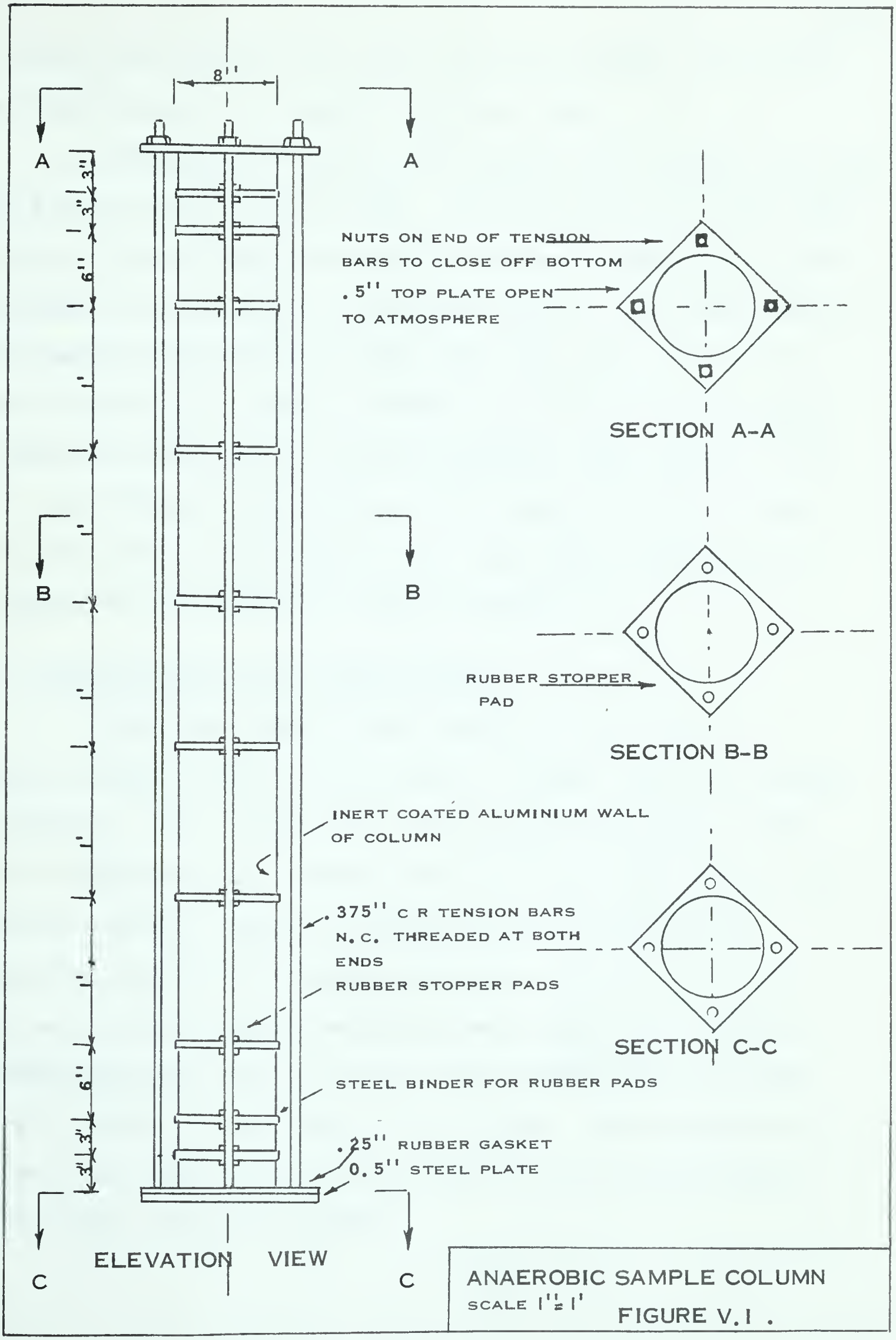
Investigations into the survival of S. typhi in sewage oxidation ponds have been very scarce. Only one such test is mentioned in "Emergency Land Disposal of Sewage" by Gillespie (16). In this discussion he stated "a particularly striking illustration of the benefit of lagoon action in reducing typhoid germs comes from Java where typhoid is so rampant; typhoid organisms are an index organism. Engineer Riis of Java reports the country is so poor that only the cheapest forms of sewage treatment can be considered, and sewage ponds are commonly used. Raw sewage contains from 1 to 41 typhoid germs per cc., the settled sewage 1 to 6 per cc., and the pond effluent is negative for typhoid." This quotation represents the only reference to the survival of typhoid in oxidation ponds found in the literature survey.

CHAPTER V

METHODS AND EQUIPMENT

V.1 The Sampling Columns

It was decided at an early date that direct testing of the waste stabilization pond using viable typhoid organisms would cause undue health hazards in the neighboring communities. In light of this, laboratory units simulating outdoor conditions were to be designed. This would enable the testing of the prototypes with the control of many variables such as lighting and sewage loading. A column or pipe was designed as shown by FIGURE V.1. to make a completely continuous depth-sample of the pond waters. In this way the sludge at the bottom could be picked up as well as the fresher sewage at the top. Thus the columns having been returned to the laboratory would represent the pond at every depth. Oxygen penetrating the surface would not penetrate to a greater depth than it would have in the actual pond. Influence of the sludge on the bottom of the waters of the pond would be similar to the outdoor conditions also. It was decided that feeding of the anaerobic column-sample would be made through small portions of sludge with strengths equivalent to those of the sewage and sludge entering the anaerobic lagoon. The columns were so designed as to afford the sampling of the columns for the inoculation of the columns through portholes. These portholes were simply holes drilled through the aluminium pipe and covered by two strips of rubber held against the pipe by packing case binding material. The inside piece of rubber 1/4" thick was designed



ANAEROBIC SAMPLE COLUMN
SCALE 1''=1'

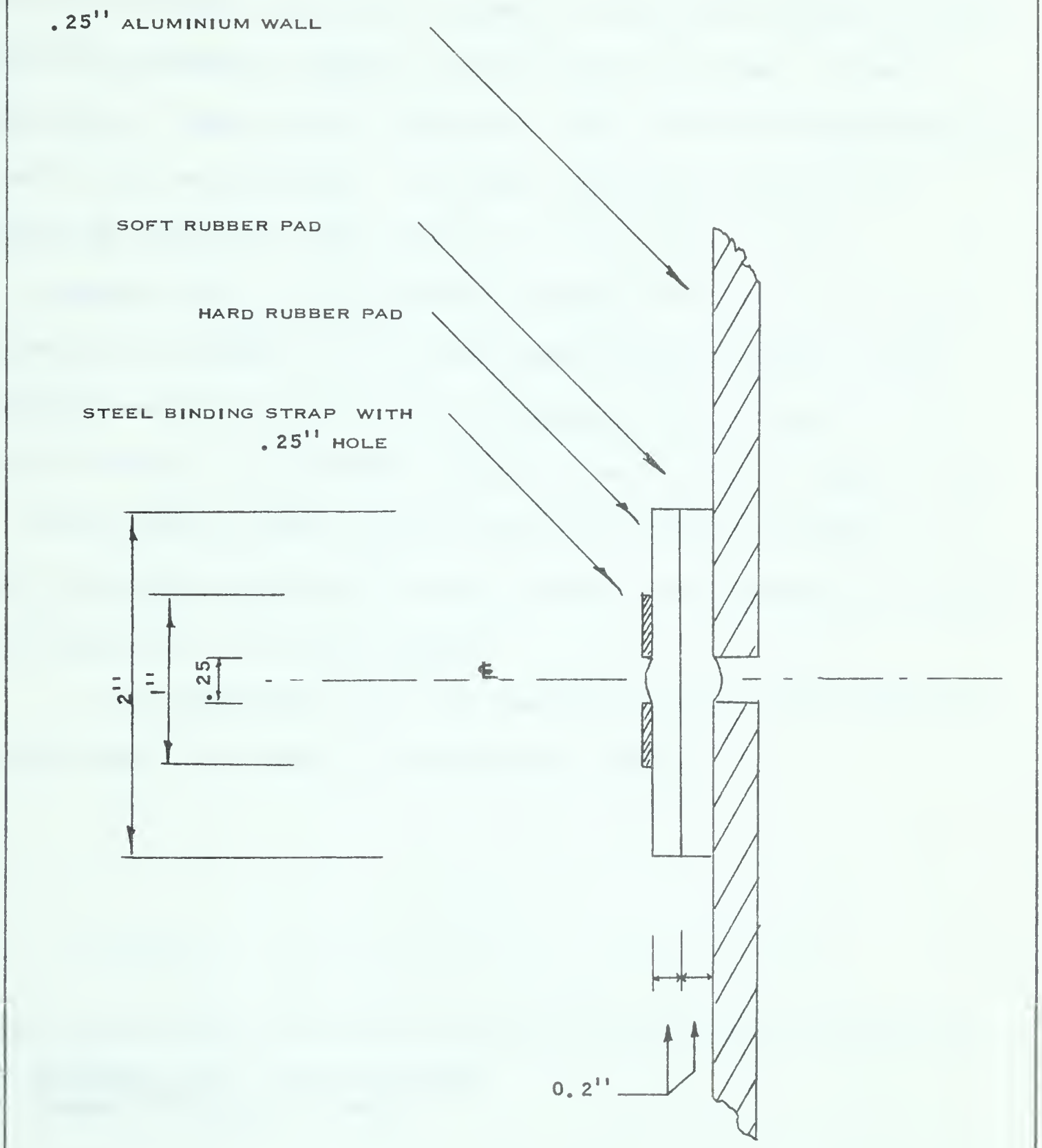
FIGURE V.1 .

to withstand the pressure of the water inside the columns. Inoculation came through the use of a standard hypodermic needle.

In this manner inoculations of bacteria could be made in accurate amounts through the sterilized needle. Extraction from the column samples was also performed through the use of the hypodermic needle for purposes of plating out the sample for culturing and counting. The bottom and top of the sample column were held firmly against both ends of the pipe by means of tension bars as shown in FIGURES III.2 & V.2. A rubber gasket 1/4" thick was placed between the pipe and base to stop leakage at that point. An 8" diameter hole in the top plate was cut to allow sunlight or artificial light into the sample column. These sample columns were 8" in diameter of 1/4" aluminium as shown in FIGURE V.2.

V.2 Internal Coating of the Sample Columns

As the sewage would corrode aluminium columns causing an undesirable metallic effect, it was necessary to apply a coating to protect the aluminium. Such a coating came in the form of TK-21 epoxy coating applied by TUBE-KOTE Inc. Normally used for storage tank interiors and industrial corrosion control it offered superior bonding, chemical temperature and physical resistance properties. It was a chemically cured epoxy coating with outstanding bonding strength and flexibility. The TUBE-KOTE process was to sand blast the aluminium piping and apply the TK-21 coating to a thickness of 10 - 20 mils.. The coating was of a rust color and smooth, and at the completion of the test there was no visible defect in the epoxy coating.



INOCULATION PORTHOLE
SCALE 1 '' = 1 ''

FIGURE V. 2.

V.3 Lighting Fixtures

Fluorescent* and incandescent+ lamps were arranged above the columns to illuminate the sewage surfaces in simulating outdoor lighting. These were supported by fixtures hanging above the column as shown in FIGURE III.2. There were two fluorescent tubes and three incandescent bulbs in each of the two fixtures. The fixture height was adjustable for purposes of varying the light intensity on the sewage surfaces.

Although several meteorological stations have been set up across the Province of Alberta by the federal Meteorological Branch, average intensities and their corresponding wavelengths over the summer months were not available in sufficient quantities and detail for effective use.

The following FIGURE V.3 taken from (35) shows the comparison in output according to wavelength between outdoor conditions and the Sylvannia 'very high output' fluorescent tubes.

The incandescent bulbs were employed to make up the deficit of red and orange wavelengths in the simulate d design.

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* Sylvannia, 48", V.H.O. Daylight
\+ General Electric, 50 Watt.

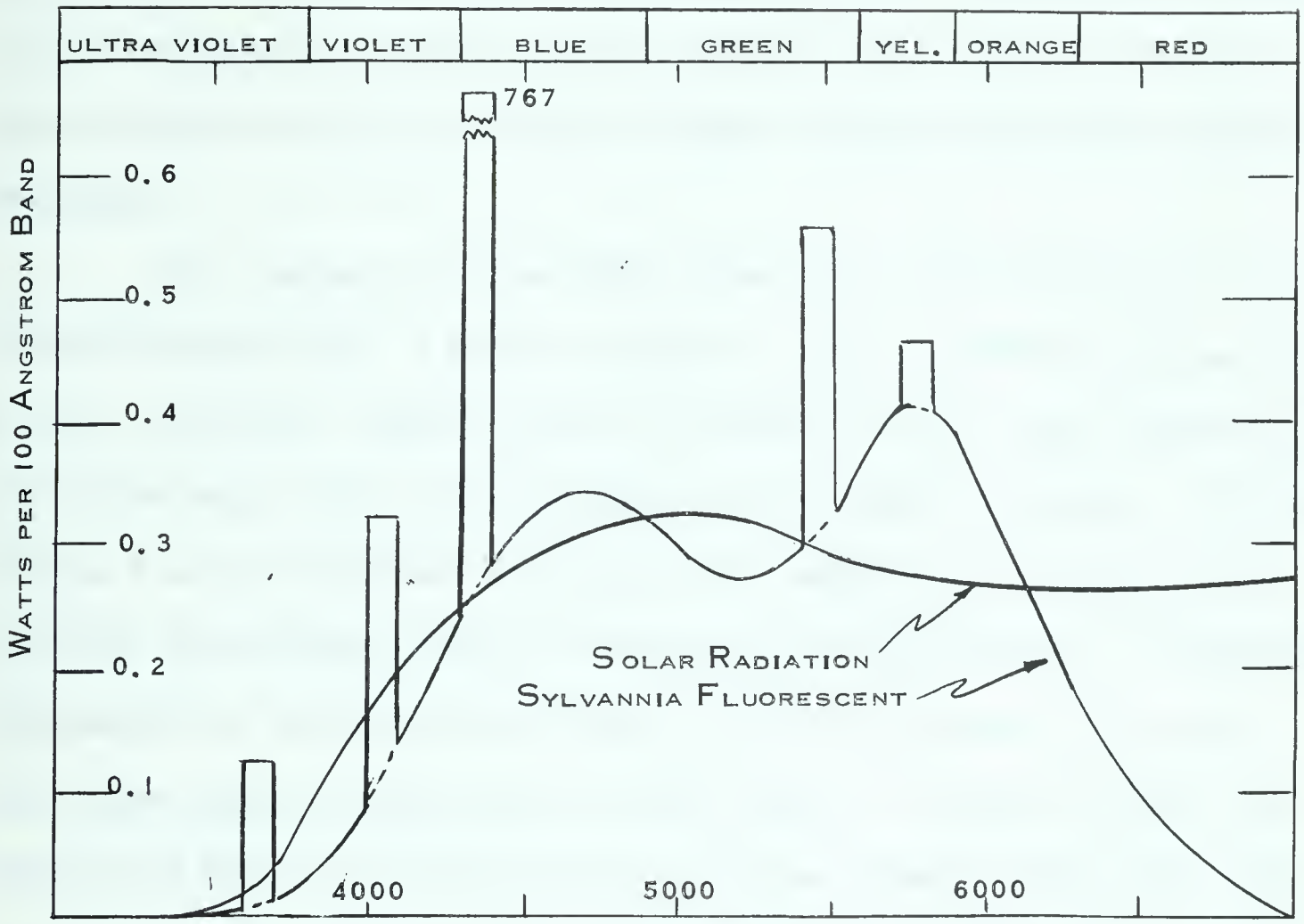

FIGURE V.3

SYLVANNIA DAYLIGHT FLUORESCENT AND SOLAR RADIATION

ENERGY VS. WATTS PER 100 ANGSTROM BAND

V.4 Nutrient Addition

In order to maintain the columns at a constant chemical and microbiological level nutrient in the form of sterilized sewage was added to the columns. As the sewage entered the long detention ponds in a settled state, the nutrient (for the aerobic columns) was taken from the Edmonton Sewage

Treatment Plant following its first settling tanks. The sterilized sewage for the much heavier loaded anaerobic columns came in the form of raw sludge taken from the Main Edmonton Sewage Treatment Plant after primary settling.

The loading of the aerobic columns was 50 lbs. BOD per acre of surface area per day. A gravity system as shown in FIGURE III.2 was devised to feed the aerobic sample columns. However, the small taps emitting the sterilized sewage soon became plugged and the addition stopped. Filtering through a number 2 filter* followed by the coagulation by autoclaving proved to be the best method to extract the larger particles. Finally the raw sewage was autoclaved as it came into the laboratory, filtered, stored and then autoclaved again before addition. Further trouble was encountered when the lines leading to the tap became plugged with algae and other forms of high plant life, sterilization of the whole apparatus then became necessary. Even after this, nutrient addition was maintained at a constant level only through daily adjustment.

The anaerobic columns were loaded with raw sterilized sewage sludge from the Edmonton Main Sewage Treatment Plant. The sludge was pulverized, autoclaved and added in proportions of 6000, 4000, 2000 and 500 lbs. BOD per acre of surface area per day. The volumes of sludge added averaged 465, 310, 155 and 39 millilitre per day. This provided a safe method of nutrient addition being both reliable and reasonably accurate. Accumulation of sludge on the bottom of the anaerobic columns was not noted to be excessive. Nutrient addition to the aerobic columns

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* Whatman Number 2 filter - W. and R. Balston, Ltd.

proved unsatisfactory to the extreme. It was decided that in view of the fact that loading rates could not be controlled to the columns daily adjustments to the rate of 50 lbs. BOD per acre per day would be maintained if possible in only one of the columns.

V.5 Stirring Mechanism

As shown in FIGURE III.2a stirring mechanisms were maintained only on the aerobic columns. It was felt that the addition of the nutrient to the anaerobic columns was enough to simulate outdoor mixing conditions. Following the addition of air to the aerobic columns, the stirring mechanism was no longer considered necessary.

V.6 Sampling of the Pond

A preliminary test in sampling with a test column was performed at the Stony Plain, Alberta anaerobic pond through the use of a drag-line and a small row boat. Following the setting up of equipment beside the pond, the column was attached to the drag-line bucket. The tension bars attached to the base plate and rubber gasket were lowered into the pond and let sit on the sludge at the bottom for a period of 3 minutes. It was quickly noticed that the base plate was sinking and a line was attached to the tension bars at the surface. Great difficulty was encountered in straightening the tension bars so that the column or pipe could be lowered to the base plate thus obtaining a representative sample of the pond waters. All too soon, both the base plate and column began to sink below the surface. It was only through climbing onto the drag-line bucket itself that the author was able to recover the equipment and lower the aluminium pipe. Through great effort, the bolts and top plate were tied down onto the tension

bars sealing off the bottom. The column was then hoisted out of the pond and placed in the truck for transportation to the University. Although a satisfactory representative sample of the pond was obtained, the author seemed to be rather prone to an unhappy fall into the pond. An adaptation of the sampling procedures was then made. In the new design through the use of a pulley system, a crane was able to lift the tension rods and base plate as well as the column as shown in FIGURE III.1a. The pulley system afforded the additional feature of being able to raise or lower the column inside the tension bars while both were being hoisted by the crane. In this way the two were lowered into the pond for the final sampling. The base plate being held at a distance of 4 feet from the pipe reached the sludge first and was allowed to settle into the sludge at the bottom and remain there for a period of approximately two minutes. The pipe was then easily lowered by the pulley onto the base plate. While the crane held the unit, the top plate was bolted down onto the column by the nuts and tension bars causing the bottom gasket to seal off the column against the base plate. The procedure is illustrated by the FIGURE III 1d. The sampling of the aerobic pond was similar to that of the anaerobic. Following the sampling the columns were transported and located in the micro-biology department of the University of Alberta, Edmonton. Complete chemical analyses of the pond site were made at the time of sampling. Four - 7 foot anaerobic sample columns and four - 4 foot aerobic columns were used. The sampling of ponds at Camrose, Alberta took place on July 22, 1964 at 11:00 a.m. The anaerobic columns were sampled near the influent entrance as shown in FIGURE VI.1 with a view to acquiring a 7 foot sample depth with 6" to one foot of raw sewage sludge. The aerobic

columns were sampled near the effluent exit of the fifth pond.

V.7 Bacterial Enumeration Techniques

There are many methods to enumerate bacterial cells in a given sample of water, amongst which are the total cell count using the Petroff Hauser bacteria counting chamber, the viable count by solid media and the turbidity count. The latter two proved to be the most reliable for the counting of the extraction samples and the inoculation samples respectively. Due to the very nature of the bacterial cell, it is possible to spread a small portion (0.1 to 1.0 ml.) on the surface of a solid media containing the necessary nutrients for the bacterial metabolism and obtain an accurate count of the viable bacteria in the sample. Through binary fission (34) the bacterial cell multiplies normally forming a mass or colony of cells after an incubation period of 24 hours at a temperature of 37°C. It is possible to see these colonies as small spots on the agar plate as shown in FIGURE V.4. Each colony is accepted to represent one original bacterial cell. Providing that there are no fewer than 30 nor more than 300 colonies per plate, it is possible to obtain an accurate count of the viable bacteria in the sample. There are media which are selective to certain specific organisms or types of organisms. These have been used in the enumeration of the typhoid and Serratia bacilli death curves.

The method for the enumeration of bacteria through the use of turbidity or optical density numbers provides for an approximate enumeration of the bacteria having been grown in a specific liquid medium. This is generally a viable test as the non-viable organisms settle to the bottom of the suspension, the O.D. measurements are made within a day of the inoculation. If curves relating the number of bacteria to the optical

density number are obtained for specific bacteria in a liquid medium, a simple extrapolation of the results may determine the concentration of those bacteria in that medium. This procedure was followed for purposes of estimating the number of bacteria being inoculated into the sample columns. The advantage of this is that the long incubation period of the plate count is eliminated. If the incubation period were required, it would be difficult to hold the new bacteria suspension at the tested concentration before inoculation.

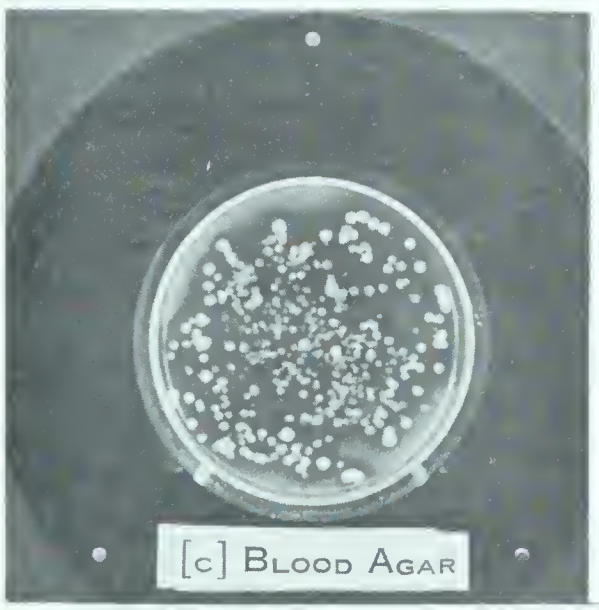
The total cell count using the Petroff Hauser bacteria counting chamber was not applicable to this project as it requires a time-consuming count of the individual cells through the microscope. A total count includes the non-viable bacteria. Enumeration of specific bacteria such as S. typhi and S. marcescens would have been impossible under these circumstances.

V.8 The Selection of Solid Medium for Isolation of S. typhi

Several media were suggested for the isolation and count of S. typhi amongst which were MacConkey agar, Salmonella-Shigella agar, Bismuth Sulphite agar, Eosin-Methylene-Blue agar, Desoxycholate agar, and Desoxycholate Citrate agar (10).

A sample of raw settled sewage was taken from the Edmonton City Main Sewage Treatment Plant and inoculated with 2×10^5 S. typhi bacilli per ml. These were plated out on nutrient agar, methylene blue agar, salmonella-shigella agar, Bismuth Sulphite agar, MacConkey's agar and blood agar plates in proportions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} mls. The plates were incubated at 37°C for 48 hours and the results shown in FIGURE V.4 & 5.

MacConkey agar is recommended for the isolation of Gram-negative



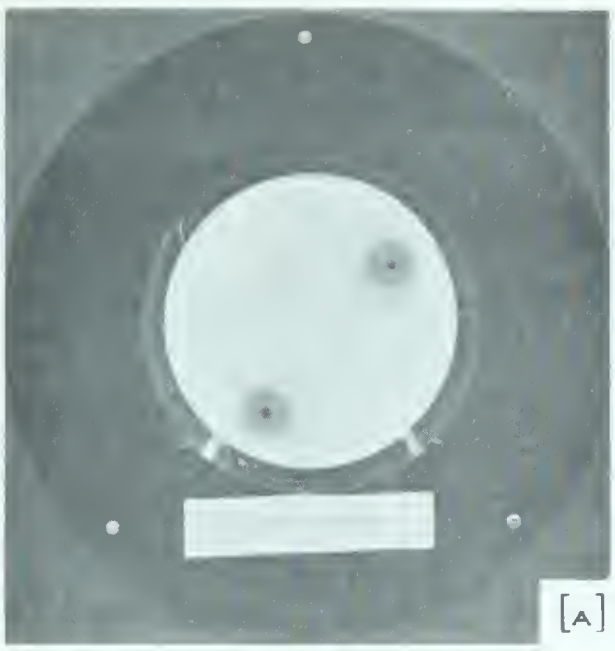
Settled Sewage Inoculated with
S. typhi on Various Media
FIGURE V. 4

pathogens such as the organisms causing all types of dysentery, paratyphoid and typhoid. The lactose fermenting organisms such as the coliforms are differentiated from the non-lactose fermenting organisms by their colony colour which is brick red as compared with the transparent non-lactose fermenting organisms' colonies. S. typhi being a non-lactose fermenting bacilli, appears as a small transparent area which may be accentuated by transmitted light. It was found however, as shown by FIGURE V.4a, that a multitude of colonies, although red, tended to blot out the S. typhi when the numbers of S. typhi were comparatively small.

The salmonella-shigella agar was tested by the same process. The results being the differentiation of the enteric pathogens, which were largely Gram-negative and non-lactose-fermenting. In particular, the Salmonella and Shigella colonies form transparent or translucent uncoloured, smooth colonies. Although this medium differentiated the non-lactose fermenting organisms to a greater extent than did the MacConkey's, other common organisms (10) than the Salmonella and Shigella were not inhibited.

The blood agar and nutrient agar plates are shown in FIGURE V.4 to give comparison to the differentiating media. 0.065 gm/l of methylene blue was added to the liquid nutrient agar before autoclaving for purposes of differentiating the Gram-negative from the Gram-positive organism. The results show an inhibition of the Gram-positives in comparison with the nutrient agar plate. Although this proved useful in the isolation of Serratia marcescens little differentiation of S. typhi was obtained.

The differential media Bismuth Sulphite was discovered by Wilson and Blair (39). Through its modified version it has proved the most often used in research of this nature. In view of FIGURE V.5 Bismuth



[A]

[B]

[C]

[D]

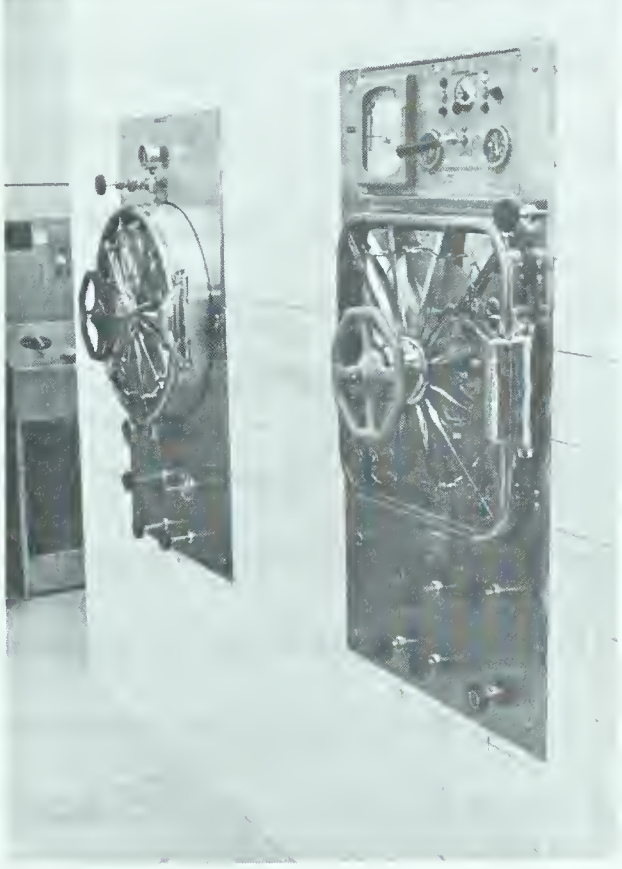
[E]

[A] Plate Inoculation, 10^{-5} ml.
[B] " " 10^{-4} ml.
[C] " " 10^{-3} ml.
[D] " " 10^{-2} ml.
[E] " " 10^{-1} ml.

Settled Sewage Inoculated with S. typhi cultured on Bismuth Sulphite Agar FIGURE V. 5

[A] Colony Counter and Plates

[B] 6° C. Refrigerator



[C] Autoclaves

Colony Counter, Refrigerator and Autoclaves

FIGURE V. 6

Sulphite agar was chosen as the most effective media for the isolation of S. typhi. Upon this medium the colonies of S. typhi grow uninhibited. They form circular, jet black, well-defined colonies, surrounded by a brown tinge approximately twice the size of the colony. The individual colony has a metallic sheen and is one to four mm. in diameter on the surface. Plates having 300 to 1000 colonies exhibit a characteristically dark background, being surficial and brown. Above three thousand colonies the plate may appear to have large clear areas(10). This effect is due to the interference between colonies not allowing for the development of the brown background as illustrated by FIGURE V.5e. The coliform group is completely inhibited on this medium as well as a large proportion of the dysentry-causing organisms. Paratyphoid A and B, S. typhimurium, S. Choleraesuis and Proteus morganii are not inhibited by the medium but form colonies easily differentiable from those of S. typhi (10).

Green and Beard (20) used Bismuth Sulphite in studies of 'The Survival of E. typhi in Sewage Treatment Plant Processes.' In these they state that there is "little to no interference from other organisms when Bismuth Sulphite agar is used. Great care must be taken in preparing the agar for use as its differentiating qualities are easily lost through over heating or improper storage." As indicated by the FIGURE V.5 the above declarations were verified. Bismuth Sulphite was chosen as the most effective medium in the isolation and counting of S. typhi.

V.9 The Selection of Media for Isolation of Serratia marcescens.

Due to the pigmentation of the Serratia bacillus the colonies display a brilliant red colour on a nutrient agar plate. Further differentiation was found to be necessary only in the case of small

concentrations of S. marcescens compared to other bacteria capable of growing on nutrient agar plates. In such a case, 0.065 gm. of methylene blue in its crystalline form was added to each litre of nutrient agar prepared. The purpose of such a preparation was to inhibit the growth of Gram-positive organisms on the plate. This proved quite adequate for the isolation of Serratia marcescens at low numbers per ml.

CHAPTER VI

TEST OBSERVATIONS

VI.1 Chemical Analyses

A preliminary pond sampling on the first anaerobic pond at Stony Plain, Alberta was carried out on June 22, 1964. The column used was eight feet in height being otherwise represented by FIGURE V.2. A successful sample was obtained although slight modifications were found necessary. Future columns were to be seven feet in height for the anaerobic pond and four feet for the aerobic. The portholes and base plate seal as described in CHAPTER V.1 proved to be wholly successful. Some difficulty was encountered in the handling of the column while it was in the pond, a pulley system was devised to overcome this problem.

Throughout the ensuing month columns were fabricated for the final sampling of the Camrose, Alberta ponds.

Shortly after the preliminary test on the Stony Plain pond the town removed the sludge from the bottom of the sampled pond. Another choice of pond system was thus required. The Camrose system offered easily accessible ponds in which only small quantities of whey wastes were treated. Whey wastes are not desirable in a study of this nature as they are very high in B.O.D. and cause large fluctuations in pond conditions. The Camrose anaerobic pond was surveyed for depth and depth of sludge in order to design column heights. Four seven-, and four four-foot columns were built to sample the anaerobic and aerobic ponds respectively. These were transported by truck to the Camrose ponds in the early morning of July 22, 1964.

Sampling locations as shown in FIGURE VI.1 were chosen for their accessibilities by crane and their representation of the widely varying conditions found in the ponds. The method of sampling has been described by CHAPTER V.6. Both the anaerobic and aerobic ponds were sampled and tested on site for their alkalinities, pH's, temperatures and dissolved oxygen contents at all depths. The anaerobic pond was further tested for its B.O.D.'s. The results of these analyses are described by TABLES VI.1 and 2 and FIGURES VI.2 to 6. The eight columns were returned by truck to the University of Alberta and placed in the Department of Microbiology for further testing. These columns were differentiated by naming them aerobic columns A, B, C, and D and anaerobic columns A, B, C, and D in order of increasing loadings from A to D.

As shown in TABLE VI.3 the dissolved oxygen content dropped sharply over the period of July 22 to August 6. The D.O. was abruptly raised to saturation by the addition of pumped air described in CHAPTER III.3 and through continual aeration remained near to the saturation level throughout the testing period.

Alkalinities were also measured throughout the testing period using the method suggested by "Standard Methods" (1). Twenty-five ml. portions of sewage from various depths of the aerobic and anaerobic columns were tested monthly throughout the testing period as described by TABLES 4 and 5.

The Beckman Zeromatic pH meter was also used to measure pH's of the columns at similar times as the alkalinities. The results are shown in TABLES VI.6 and 7. In this way a continual check on chemical conditions was made of the columns.

Residues (solids) were analyzed at the same depths and times as the alkalinities. These were tested and results were obtained in the forms

CAMROSE OXIDATION PONDS
FIGURE VI. 1

TABLE VI.1

ANAEROBIC POND CHEMICAL ANALYSIS July 22, 1964

DEPTH		pH	D.O. ppm	ALKALINITY*	TEMPERATURE °C	B.O.D. ppm
0		7.3	0	560	19°	268
6"		7.3	0	540	19°	237
1'		7.3	0	520	19°	254
2'		7.1	0	560	19°	230
3'		7.1	0	400	19°	230
4'		7.15	0	400	19°	242
5'		7.0	0	480	19°	Septic
6'		6.8	0	300	19°	Sludge
7'		7.0	0	290	19°	Sludge
			BOTTOM DEPTH			
Dec. 3	2'	7.8	0	589		

All analyses performed as according to (1) .

★ Total alkalinity as bicarbonate ppm as $CaCO_3$.

Tested July 22, 1964.

Observations plotted in FIGURES VI.2 & 3.

TABLE VI.2

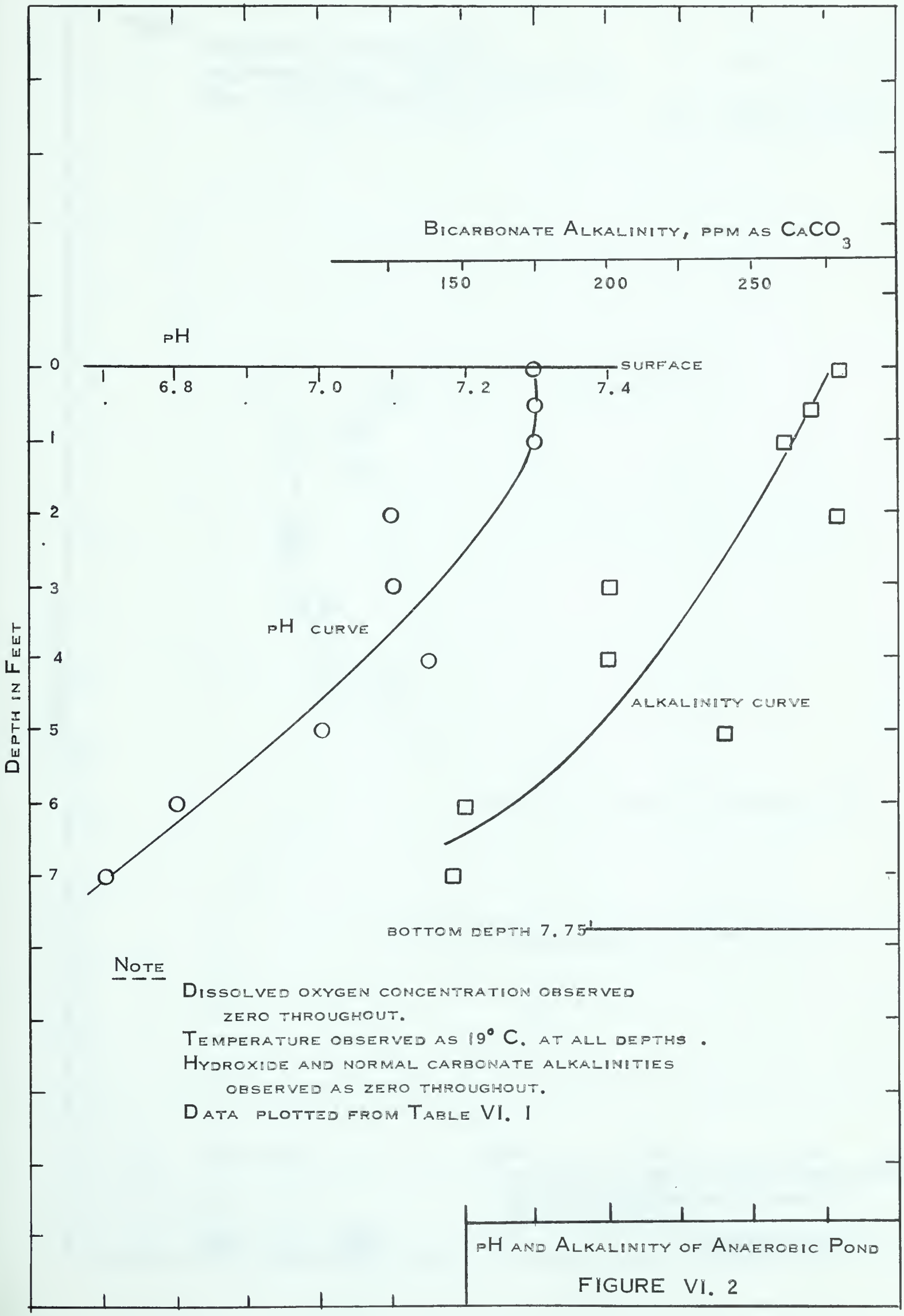
AEROBIC POND CHEMICAL ANALYSIS

DEPTH	pH	D.O.	Alkalinities ppm as $CaCO_3$			TEMP.
			CO_3	HCO_3	OH	°C
0	8.7	24.0	320	80	0	23
3"	9.1	24.6	240	140	0	23
6"	9.05	26.0	320	160	0	23
1'	9.2	26.0	120	380	0	21.5
2'	9.0	18.0	60	440	0	22
3'	8.8	18.0	60	440	0	21
4'	8.8	10.0	140	455	0	19.5
5'	8.7	6.2	140	450	0	-
6'	BOTTOM DEPTH					
Dec. 3, 2'	8.3	-	0	730	0	-

All analyses performed as according to (1).

Tested July 22, 1964.

Observations plotted in FIGURES VI.4, 5 & 6.



Note

Dissolved oxygen concentration observed zero throughout.

Temperature observed as 19° C. at all depths.

Hydroxide and normal carbonate alkalinities observed as zero throughout.

Data plotted from Table VI. 1

pH and Alkalinity of Anaerobic Pond

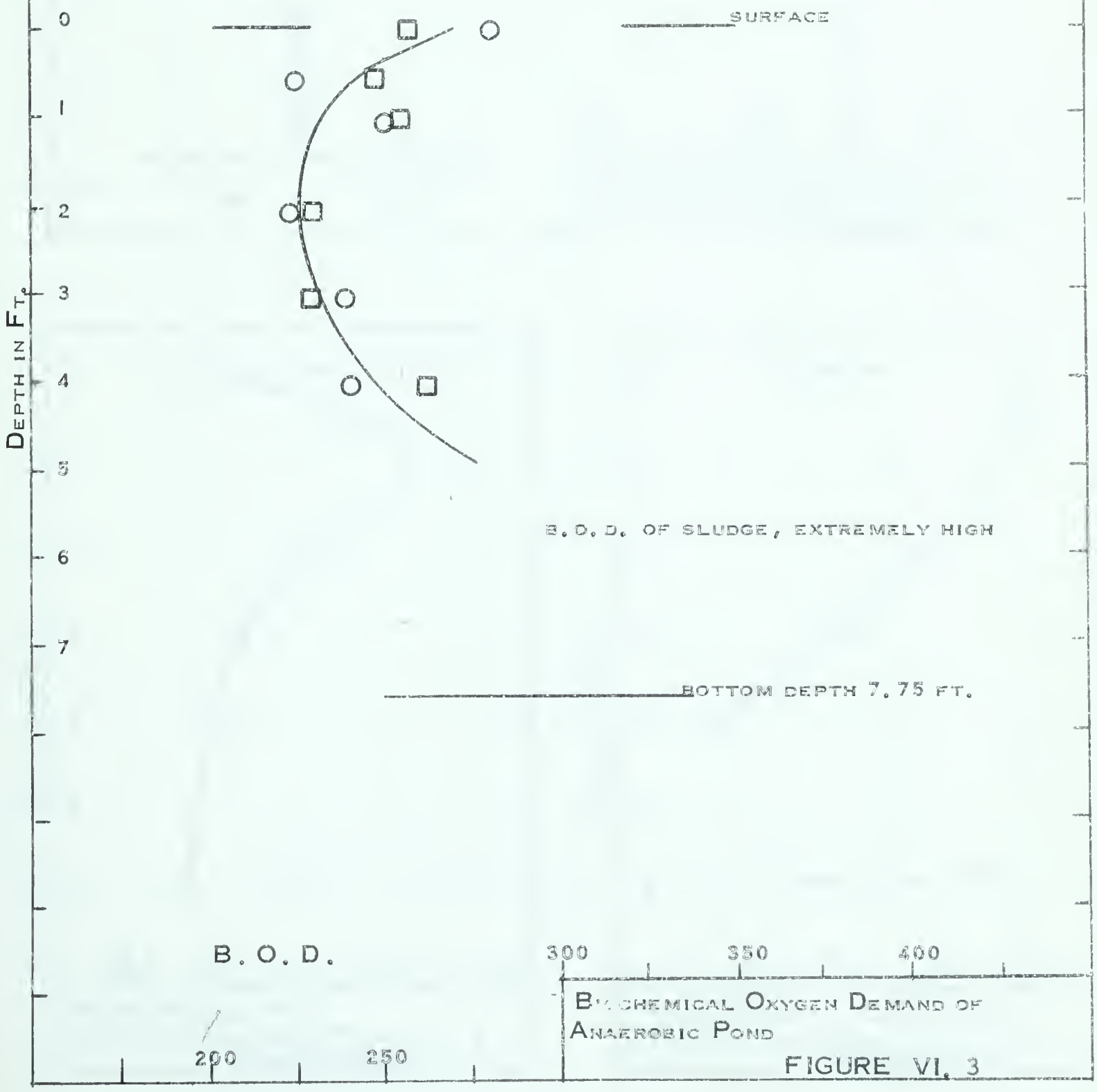
FIGURE VI. 2

Notes

Biochemical oxygen demand
incubation time, 5 days, temp, 20° C,
Data plotted from Table VI. 1

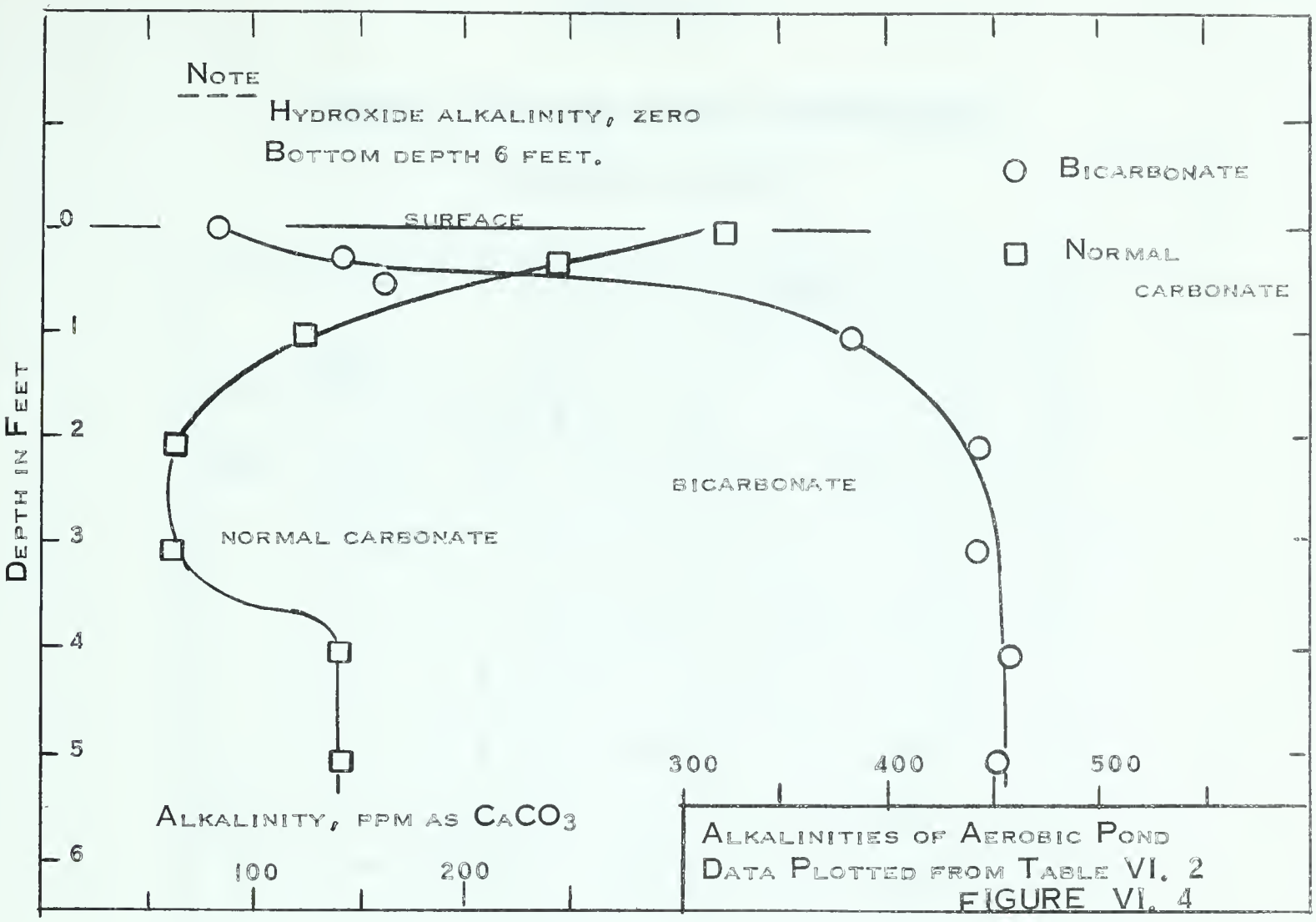
○ 3.75 ml. dilution

□ 5.00 ml. dilution

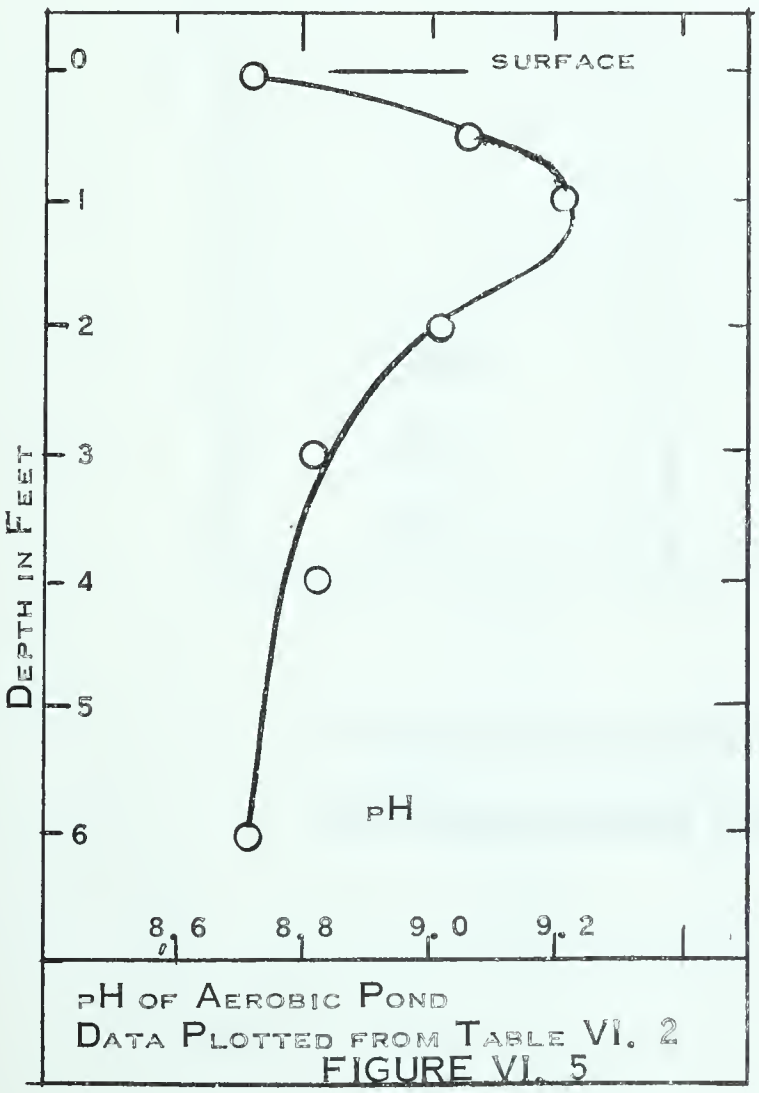


Biochemical Oxygen Demand of
Anaerobic Pond

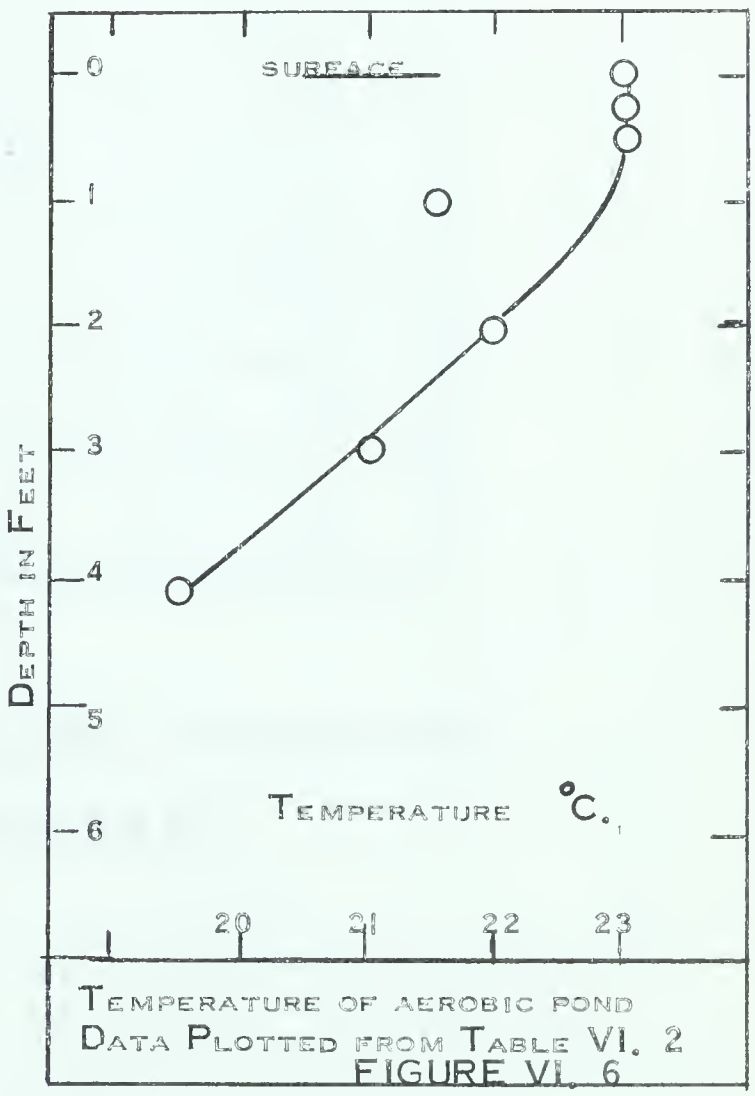
FIGURE VI. 3



ALKALINITIES OF AEROBIC POND
DATA PLOTTED FROM TABLE VI. 2
FIGURE VI. 4



pH OF AEROBIC POND
DATA PLOTTED FROM TABLE VI. 2
FIGURE VI. 5



TEMPERATURE OF AEROBIC POND
DATA PLOTTED FROM TABLE VI. 2
FIGURE VI. 6

TABLE VI.3

SURFACE DISSOLVED OXYGEN CONCENTRATIONS

IN AEROBIC COLUMNS

DATE			COLUMN			
			A	B	C	D
1964	July	22	24.0	24.0	24.0	24.0
	August	4	3.3	0.9	0.9	0.5
		5	2.9	1.6	0.3	1.5
		6	0	0.1	0	0
		8	6.0	6.9	7.4	7.0
		10	6.5	7.2	8.0	7.5
		12	6.5	7.1	7.5	7.4
		14	6.3	7.5	8.0	8.0
		15	5.7	7.3	8.0	7.3
		16	5.0	7.0	7.1	7.2
		20	5.7	5.6	6.5	6.5
		21	7.5	7.1	7.2	6.5
	Sept.	7	6.6	6.4	6.6	6.7
	Oct.	7	5.8	6.4	6.6	6.4
	Nov.	12	6.6	-	-	-

All analyses according to (1) with modification.

All concentrations given as ppm O_2.

TABLE VI.4

ALKALINITIES IN ANAEROBIC COLUMNS

COLUMN	A	B	C	D	A	B	C	D	A	B	C	D
TOTAL ALKALINITY*	HCO_3				HCO_3				HCO_3			
DATE	September 8				October 6				November 13			
DEPTH												
0												
3"		900		980						1384		1480
6"				962					1220		1664	
1′	982	902	956	1000								
2′	940	940	965	965	940	1000	1100	1220	1280	1360	1688	1672
3′		880	965									
4′	925	880	958	960					1260	1420	1668	1640
5′						1000	1050	1270	1220			
6′												

* Alkalinities as ppm $CaCO_3$, all bicarbonate.
All analyses according to (1).

TABLE VI.5

SURFACE ALKALINITIES* IN AEROBIC COLUMNS

COL.	August 20			August 21			Sept. 7			Sept. 15			Oct. 7*			Nov. 12°			Nov. 13		
	OH	CO_3	HCO_3	OH	CO_3	HCO_3	OH	CO_3	HCO_3	OH	CO_3	HCO_3	OH	CO_3	HCO_3	OH	CO_3	HCO_3	OH	CO_3	HCO_3
A	0	20	303	0	24	328	0	88	212	0	64	230	0	64	263	0	24	308	0	82	268
B	0	34	329	0	44	289	0	32	176	0	40	160	0	40	184		-			-	
C	0	40	294	0	70	270	0	28	292	0	48	244	0	112	168		-			-	
D	0	60	312	0	40	272	0	56	264	0	40	220	0	48	226		-			-	

* Taken at Depth 1'
° " " " 6"

± Alkalinities as ppm. $CaCO_3$.
All analyses performed according to (1).

TABLE VI.6

SURFACE pH IN AEROBIC COLUMNS

DATE	July 22	Aug. 15	Aug. 16	Aug. 20	Aug. 21	Sept. 7	Sept. 15	Oct. 7	Nov. 12	Nov. 13
COLUMN										
A	7.3	8.5	7.9	8.5	8.5	9.5	9.25	9.0	8.7	8.8
B	7.3	8.7	8.3	8.7	8.8	8.7	9.0	9.0		
C	7.3	8.2	7.1	8.9	9.0	9.1	9.0	9.6		
D	7.3	8.4	8.1	8.9	8.7	9.1	8.9	9.0		

All analyses performed according to (1 .

TABLE VI.7

pH LEVELS IN ANAEROBIC COLUMNS

COLUMN	A	B	C	D	A	B	C	D	A	B	C	D
DATE	September 8				October 6				November 13			
DEPTH												
0												
0 - 3"		6.7								8.2		8.1
0 - 6"									7.5		8.2	
1' - 0	7.1	6.7	6.7	6.8								
2'	7.1	6.6	6.7	6.7	7.5	7.0	7.0	7.0	8.3	8.3	8.3	8.2
3'	7.1	6.7	6.5	6.7								
4'	7.0	6.7	6.6	-					8.2	8.3	8.3	8.2
5'	-	-	-	6.7	7.3	7.0	7.0	7.0				

All analyses performed according to (1).

of filterable volatiles, non filterable volatiles, filterable non-volatiles and non filterable non-volatiles. The procedure and equipment used was as suggested by "Standard Methods" (1). The resulting data is given in TABLE VI.8 & 9.

VI.2 Observations of Tests Related to Serratia marcescens

In order to observe the death rate of Serratia marcescens in the aerobic and anaerobic columns, suspensions of the bacteria were to be added to the sewage. The method used is described in CHAPTER V.7. Inoculations were made by hypodermic needle and syringe through the portholes into the columns.

In order to approximate the number of bacilli per ml. being inoculated into the columns, the optical density number of the number of bacteria per ml. in suspension as described by TABLE VI 10 and FIGURE VI.8 was to be established. The nutrient used in this case was tryptocase soy broth, as Serratia marcescens is known to grow rapidly in this medium. Volumes of 10 ml. were inoculated and incubated at 37°C. These were shaken by hand and tested for optical density and number of bacteria per ml. every hour. Using the standard serial dilution techniques as described by Myers (29) 0.1 ml. portions of serially diluted samples were plated out on nutrient agar plates every hour for counting purposes.

The instrument used in obtaining optical density numbers was the Bausch and Lomb Spectronic 20 spectrophotometer. Optical density is described as

$$O.D. = \log_{10} \frac{1}{T} \qquad (4)$$

where 'O.D.' is optical density and 'T' represents the transmissability of the suspension. Readings were made by light of 540 Angstroms in wavelength. The purpose of using optical density numbers in place of the transmissability

TABLE VI.8

SOLIDS CONTENT (RESIDUE) mg/1. IN ANAEROBIC COLUMNS

DATE	October 6								November 12											Dec.3
COLUMN	A	A	B	B	C	C	D	D	A	A	A	B	B	C	C	C	D	D	D	Camrose
DEPTH IN FT.	2	5	2	5	2	5	2	5	6"	2'	4'	3"	4'	6"	2'	4'	2'	5'	3"	
nonfilterable volatiles	52	40	124	128	176	206	168		80	32	116	64	16	-	4	72	28	44	40	128
nonfilterable non-volatiles	76	88	0	0	0	0	0		32	20	0	207	19	-	16	204	0	12	16	76
filterable volatiles	460	464	444	708	620	424	708	692	328	412	388	688	588	792	677	513	917	1022	-	224
filterable non-volatiles	1212	1092	1092	1036	1152	956	1384	1456	1112	1308	1520	2040	1340	1680	1025	1880	1480	1480	-	744

All analyses according to (1).

TABLE VI.9

SOLIDS CONTENT (RESIDUE) mg/l. IN AEROBIC COLUMNS

DATE	September 15								October 7						Nov. 12				
COLUMN	A	A	B	B	C	C	D	D	A	A	B	C	C	D	A	A	A	A	Camrose Pond #5
DEPTH	6"	3'	6"	3'	6"	3'	6"	3'	1'	3'	3'	1'	3'	3'	2'	1'	3"	3'	
non-filterable volatiles	18	0	16	0	0	8	-	0	16	0	0	14	0	0	40	30	24	4	24
non-filterable non-volatiles	12	-	8	24	0	14	-	0	18	36	34	26	30	40	34	34	38	56	26
filterable volatiles	64	54	26	32	48	66	72	68	80	82	64	58	-	94	54	52	70	-	126
filterable non-volatiles	512	464	364	386	460	466	530	554	462	420	438	422	-	486	368	424	416	-	438
TOTAL	606	-	414	442	508	544	-	622	576	538	536	520	-	620	496	540	548		614

All analyses according to (1).

[A] Analytical Balance

[B] Steam Baths

[C] Muffle furnaces

Equipment Used in the Determination of Residues FIGURE VI. 7

TABLE VI.10

SERRATIA MARCESCENS , GROWTH CURVE AND O.D.* NUMBERS

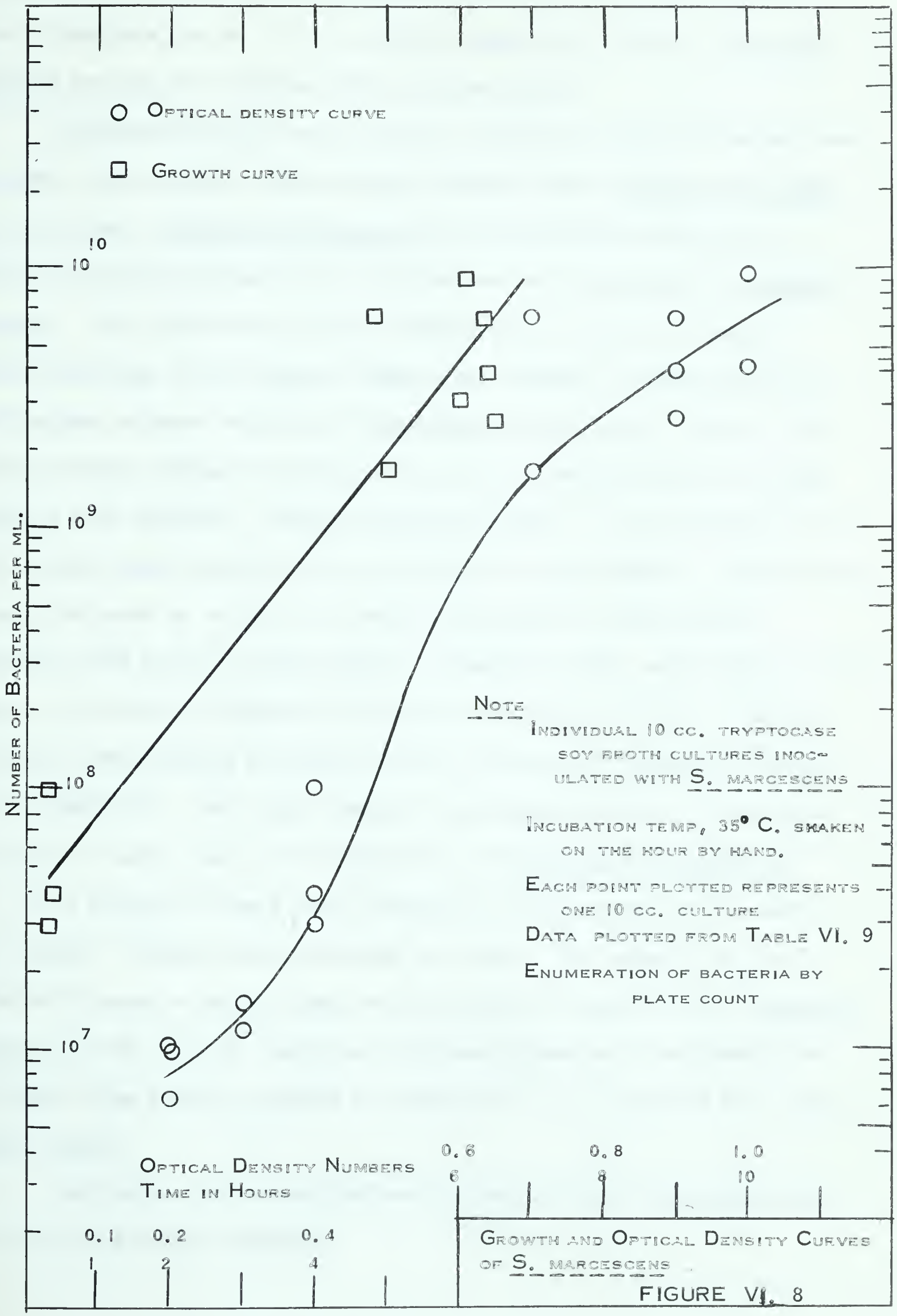
TIME HRS.	#/ml.	O.D.	#/ml.	O.D.	#/ml.	O.D.
1	10^7	0	6.3×10^6	0	10^7	0
2	1.5×10^7	0	1.2×10^7	0	-	-
3	10^8	.03	3×10^7	.04	4×10^7	.04
6	1.7×10^9	.50	-	-	6.6×10^9	.49
7	3×10^8	.56	8×10^8	.59	1.2×10^8	.64
8	6.4×10^9	.63	2.6×10^9	.65	4×10^9	.64
9	3.2×10^9	.60	-	.61	9.6×10^9	.612

All analyses made according to (29).

* O.D. - Optical Density

Incubated at 37°C in tryptocase soy broth, shaken continuously.

Observations plotted in FIGURE VI.8.



Growth and Optical Density Curves of S. marcescens

FIGURE VI. 8

was to obtain a straight line curve when plotting optical density versus number of bacteria per ml. In this way the approximate number of Serratia marcescens per ml. was obtained before its inoculation.

Preliminary batch tests on 100 ml. volumes of sewage obtained from the aerobic and anaerobic columns being inoculated with Serratia marcescens were carried out. Serratia marcescens was inoculated into the 100 ml. volumes and tested continually for the presence and enumeration of Serratia marcescens. The samples were stirred continuously to avoid the larger particles settling to the bottom. These tests enabled a testing program to be set up with advance knowledge of approximate death curves. Tests on the columns were made through the extraction of 1 ml. samples from the columns at various time intervals. Through serial dilutions .1 ml. portions of the diluted sewage were inoculated onto methylene blue agar plates. The methylene blue agar was made up in order to inhibit the growth of Gram-positive organisms on the plates. Sixty five mg. of methylene blue were added per litre of nutrient agar during its preparation before autoclaving. Use of methylene blue plates proved highly successful in the inhibition of Gram-positives as shown by FIGURE V.4. The sewage samples were inoculated onto the methylene blue plates in such a way as to obtain 30 to 300 Serratia marcescens per plate. This resulted in more than one dilution being plated out for each sewage sample.. Usually three dilutions were made. For example, if in a particular instance a sewage sample was estimated to contain 5×10^4 Serratia marcescens per ml., 0.1 ml. portions of diluted sewage would be inoculated onto plates being serially diluted to contain 10^{-2}, 10^{-3} and 10^{-4} ml. of the original sewage.

The plates, thus inoculated would then be stored and incubated at 37°C or 24 hours before counting.

Inoculations into the anaerobic and aerobic columns on October 12 and 27 respectively were made in portions calculated to produce a population of Serratia marcescens in the column sewage of 10^5 per ml. Suspensions were added throughout the length of the columns through all portholes. The addition of air into the aerobic columns caused an even distribution of Serratia marcescens throughout. A nine foot glass stirring rod was used to mix the anaerobic columns after inoculation. The glass rod was turned in the column in a wide arc about the perimeter of the columns two or three times to distribute the S. marcescens laterally. Care was taken not to stir up sludge on the bottom nor to cause excessive vertical mixing. Continuous bubbling caused by the methane producing bacteria and floating sludge was noticed throughout the testing program. The resulting death curve data is given in TABLES VI.11 to 13 and shown in FIGURES 9 to 14.

VI.3 Observations of Tests Relating to Salmonella typhi

The testing procedures for the growth curves and O.D. numbers of S. typhi A40 followed a similar pattern as those of the previously described Serratia marcescens. Two one hundred ml. volumes of nutrient broth were inoculated with S. typhi on November 4, 1964. These were incubated at 37°C while being shaken continuously. Throughout the incubation, samples were extracted from the cultures for bacterial enumeration and optical density measurements.

The enumeration was made through standard serial dilution and plating techniques (29). A check was made on Bismuth Sulphite agar to assure the author that there was no inhibition of S. typhi itself on this medium. Comparative plates were made of nutrient and Bismuth Sulphite agar. The results are recorded by TABLE VI.14 and FIGURES VI.15, 16 & 17.

TABLE VI.11

TEST DATA FOR THE DEATH CURVES OF SERRATIA MARCESCENS IN THE ANAEROBIC COLUMNS

COLUMN	A		B		C		D	
DEPTH	Time	#/ml.	Time	#/ml.	Time	#/ml.	Time	#/ml.
3"			0.8	3×10^3			0.9	1.1×10^3
6"	0.5	6.9×10^3	-	-	0.9	1.4×10^3	-	-
2'	0.5	10^2	0.8	2.2×10^3	0.9	3.3×10^3	1.0	1.8×10^3
4'	0.55	2.3×10^3	0.8	1.8×10^3	0.9	3.0×10^3	-	-
5'	-	-	0.9	4.1×10^3	-	-	1.0	1.5×10^3
6'	0.66	1.4×10^3	-	-	1.0	7.6×10^3	1.1	2.8×10^4
3"			3.9	3.3×10^3			3.9	3.1×10^3
6"	3.6	3×10^3	-	-	3.8	7.3×10^3		
2'	3.7	3.6×10^3	3.9	3.2×10^3	3.9	5.6×10^3	3.9	2.8×10^3
4'	3.9	2.3×10^3	3.9	1.8×10^3	3.9	4.8×10^3		
5'			3.9	3.2×10^3			4.0	2.2×10^3
6'	3.9	2×10^3			3.9	3.9×10^3	4.0	7.1×10^3
3"			13.1	2.4×10^3			13.1	1.9×10^3
6"	13.0	1.3×10^3			13.1	1.4×10^3		
2'	13.0	1.6×10^3	13.1	1.7×10^3	13.1	2.5×10^3	13.1	1.7×10^3
4'	13.0	1.8×10^3	13.1	1.6×10^3	13.1	1.7×10^3	13.2	
5'			13.2	2.3×10^3			13.2	3.0×10^3
6'	13.1	3×10^3			13.2	1.9×10^3		1.3×10^4

Time given in hours.
All analyses according to (29).
No indication of S. marcescens was observed prior to test.
Tests started, October 12, 1964.
Observations plotted in FIGURES VI.9 to 12.

TABLE VI.11 (cont'd.)

TEST DATA FOR THE DEATH CURVES OF SERRATIA MARCESCENS IN THE ANAEROBIC COLUMNS

COLUMN	A		B		C		D	
DEPTH	Time	#/ml.	Time	#/ml.	Time	#/ml.	Time	#/ml.
3"			23.9	0			24.0	0
6"	23.9	10^4			23.9	9.9×10^3		
2'	23.9	3.9×10^3	23.9	3.9×10^3	24.0	1.4×10^3	24.0	1.1×10^3
4'	23.9	1.2×10^3	24.0	1.4×10^3	24.0	1.5×10^4		
5'			24.0	8.5×10^3			24.0	$5 \times 10^{2*}$
6'	24.0	2.8×10^3			24.0	1.1×10^3	24.1	$2 \times 10^{2*}$
3"			27.1	0			27.2	0
6"	27.1	10^{2*}			27.2	0		
2'	27.1	0	27.2	20^*	27.2	0	27.2	0
4'	27.1	0	27.2	0	27.2	0		
5'			27.3	0			27.3	0
6'	27.1	0			27.3	0	27.3	0
3"			34.2	0			34.1	0
6"	34.1	0			34.1	0		
2'	34.1	0	34.2	0	34.1	0	34.1	0
4'	34.2	0	34.2	0	34.2	0		
5'			34.2	0			34.2	0
6'	34.2	0			34.2	0	34.2	0

Time given in hours
All analyses according to (29).
* Statistically insignificant but S. marcescens present, omitted from average.
No indication of S. marcescens was observed prior to test.
Tests started, October 12, 1964.
Observations plotted in FIGURES VI.9 to 12.

TABLE VI.12

TEST DATA FOR THE DEATH CURVES OF SERRATIA MARCESCENS IN ANAEROBIC COLUMNS, AVERAGED VALUES

COL. A		COL. B		COL. C		COL. D	
TIME-HRS.	#/ml.	TIME-HRS.	#/ml.	TIME-HRS.	#/ml.	TIME-HRS.	#/ml.
0.55	2.7×10^3	0.8	2.8×10^3	0.9	3.6×10^3	1.0	1.8×10^3
3.8	3×10^3	3.9	2.8×10^3	3.9	5.4×10^3	4.0	3.8×10^3
13.0	1.9×10^3	13.1	2.0×10^3	13.1	3.9×10^3	13.2	2.0×10^3
23.9	4.5×10^3	24.0	3.4×10^3	24.0	3.5×10^3	24.0	0.5×10^3
27.1	0	27.1	0	27.2	0	27.3	0
34.2	0	34.2	0	34.2	0	34.2	0

All analyses made according to (29).

No indication of S. typhi prior to test.

Tests started Oct. 12, 1964.

Observations plotted in FIGURE VI.13.

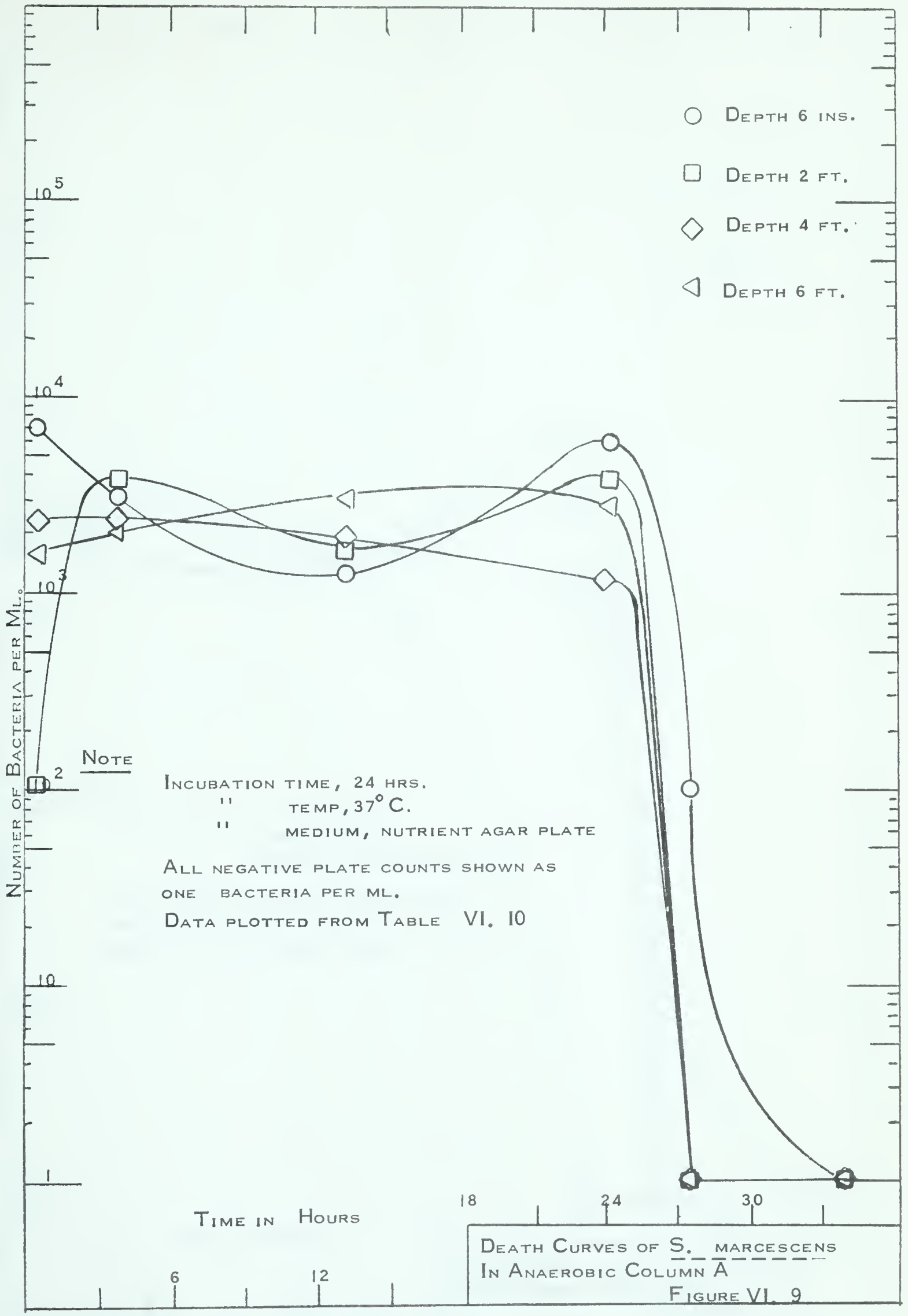
TABLE VI.13

TEST DATA FOR THE DEATH CURVES OF

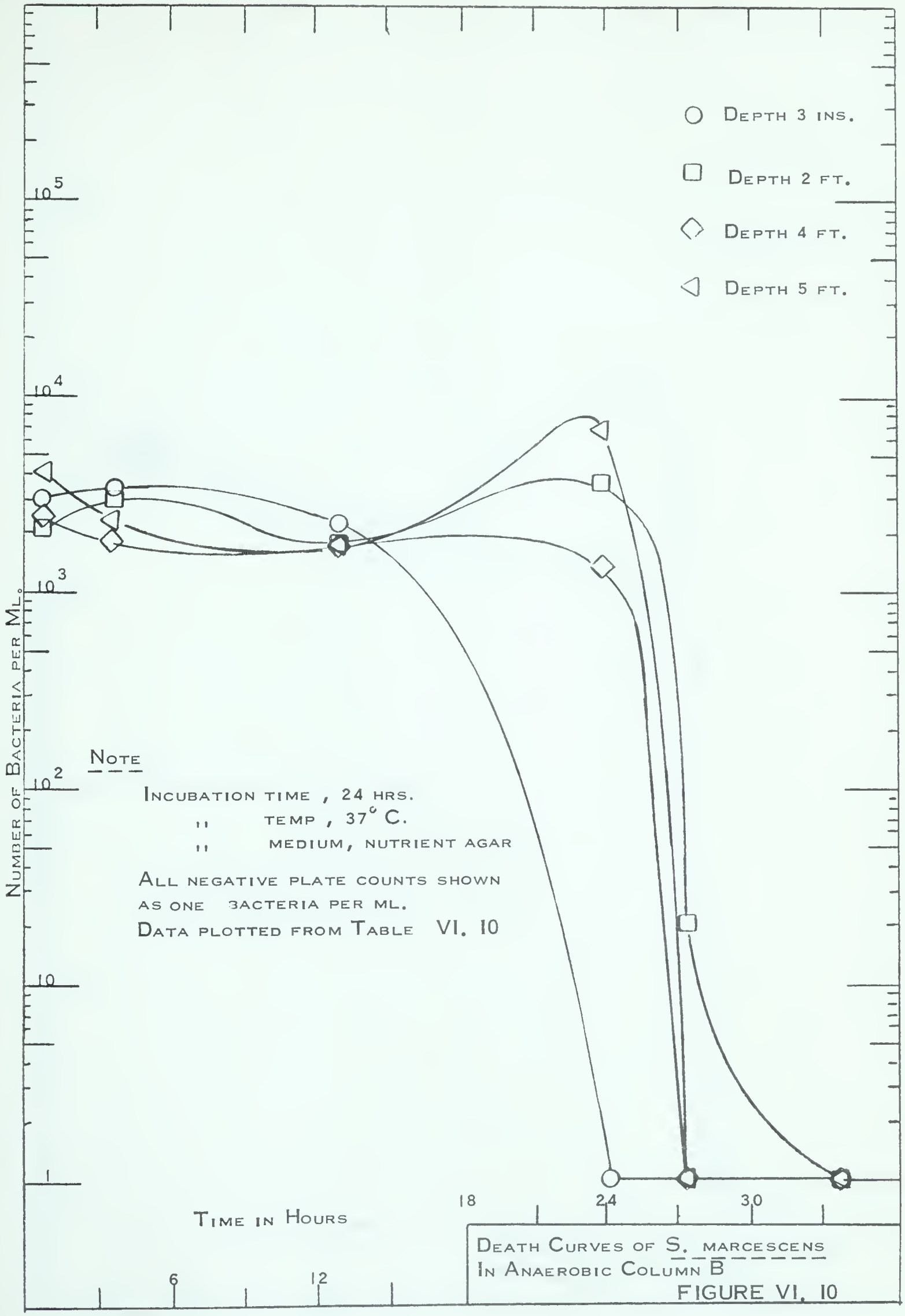
SERRATIA MARCESCENS IN AEROBIC COLUMN

DEPTH	3"		1'		2'		3'	
TIME HRS.	NAP	MBA	NAP	MBA	NAP	MBA	NAP	MBA
0.5	3.2×10^3	2.7×10^3	3.8×10^3	3.3×10^3	3.7×10^3	1.9×10^3	2.5×10^3	3.4×10^3
1.3	1.4×10^3	1.4×10^3	1.4×10^3	1.2×10^3	10^3	1.6×10^3	2.1×10^3	2.2×10^3
5.7	10^*	0	10^*	0	40^*	0	0	-
11.0	0	0	0	-	0	0	0	-
22.4	0	0	0	-	0	0	0	-
30.0	0	0	0	-	0	0	0	-

* Statistically insignificant although presence of S. marcescens observed.
All analyses performed according to (29).
Bacterial Enumeration given as #/ml.
No evidence of S. marcescens in column before test.
Tests started October 27, 1964.
Observations plotted in FIGURE VI.14.

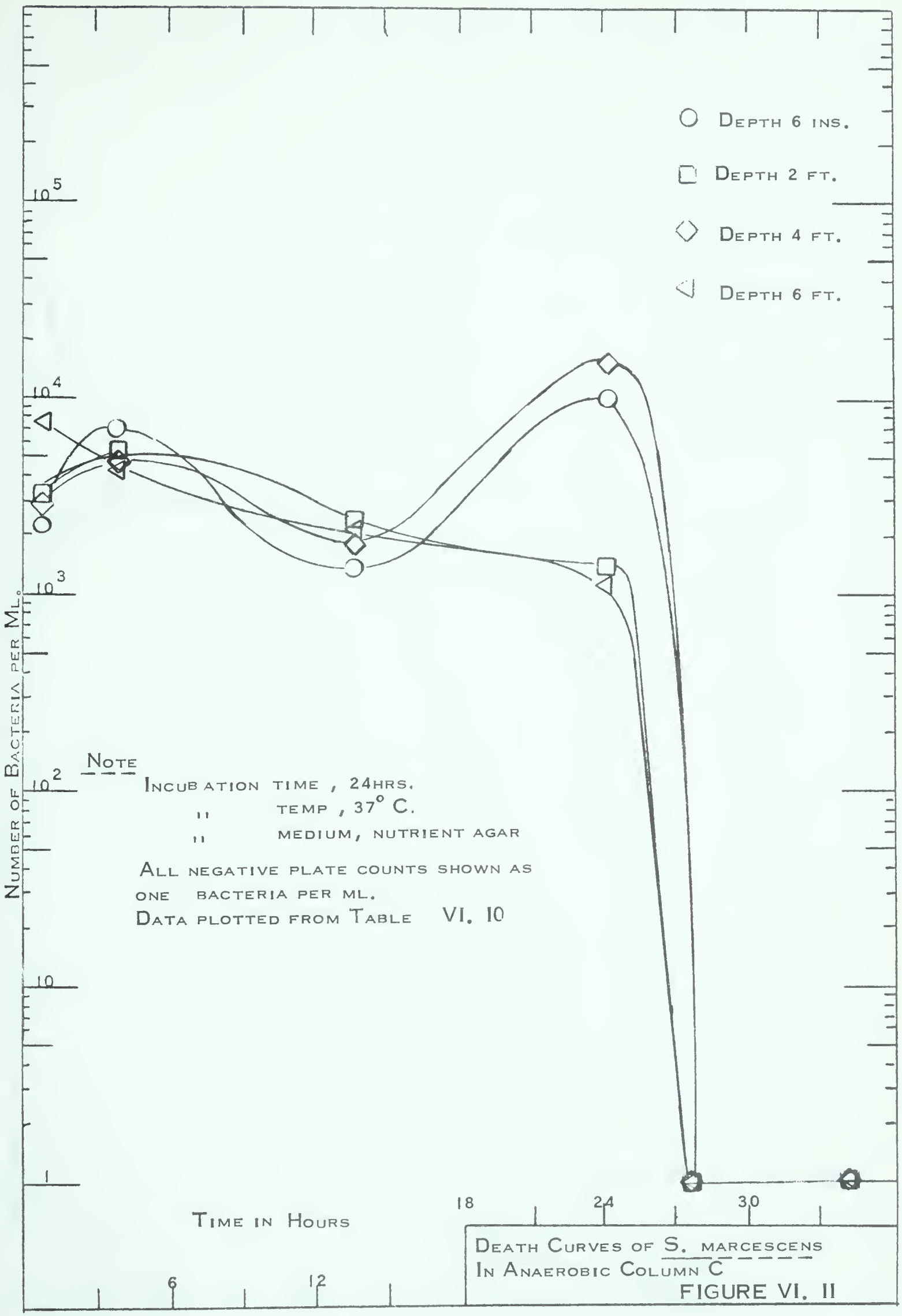


Death Curves of S. marcescens
In Anaerobic Column A
Figure VI. 9



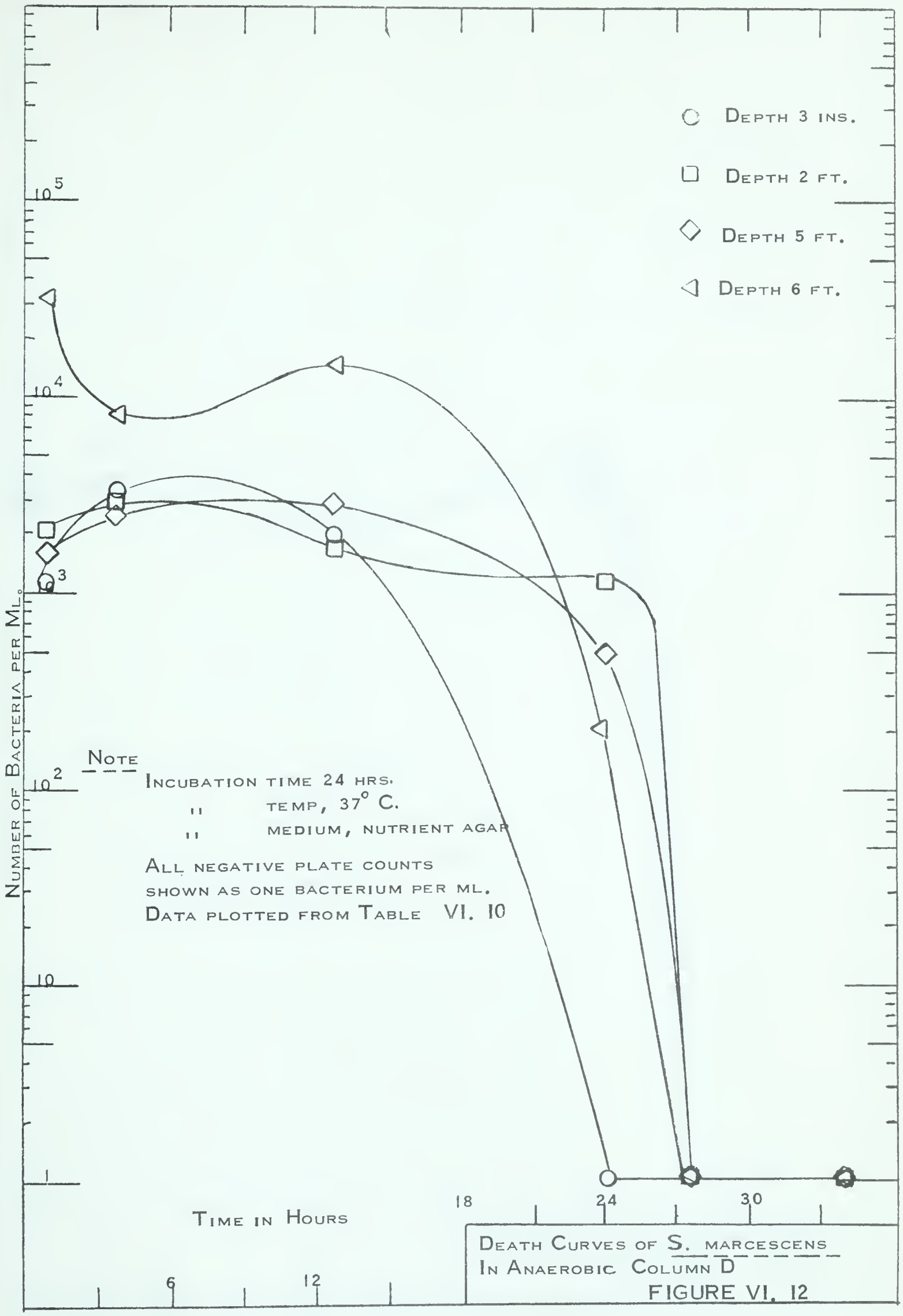
Death Curves of S. marcescens In Anaerobic Column B

FIGURE VI. 10



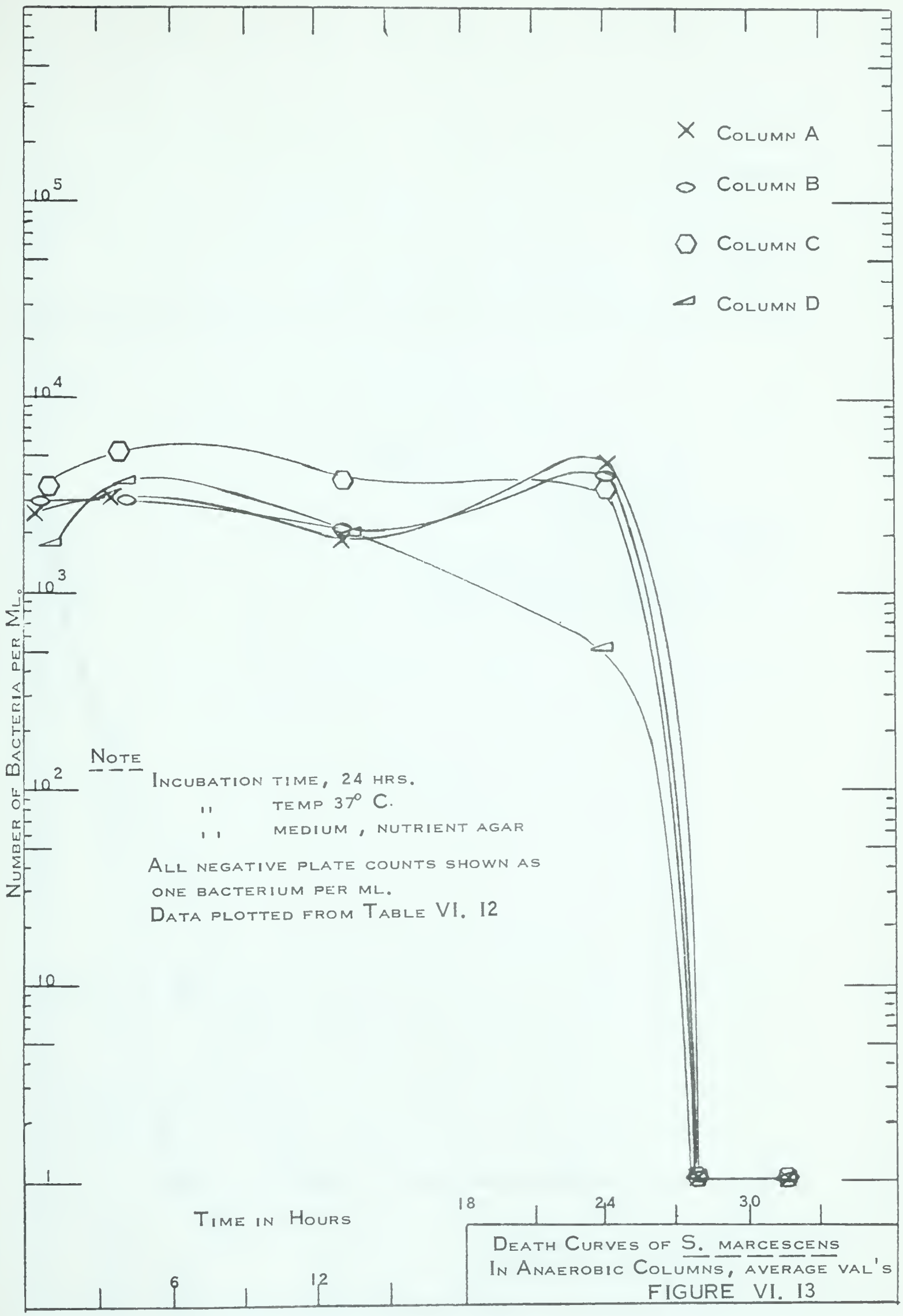
Death Curves of S. marcescens
In Anaerobic Column C

FIGURE VI. II



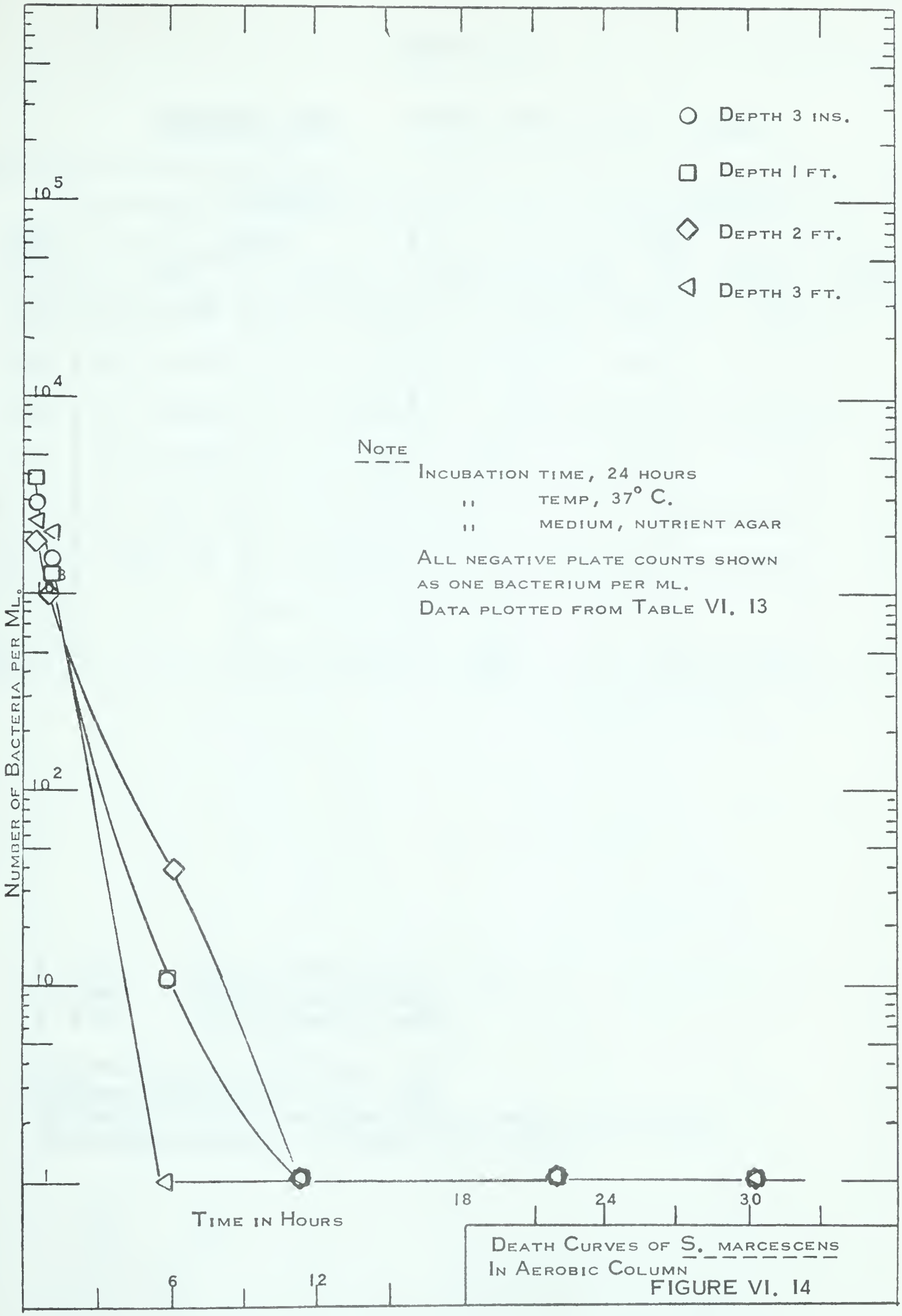
Death Curves of S. marcescens In Anaerobic Column D

FIGURE VI. 12



Death Curves of S. marcescens
In Anaerobic Columns, average val's

FIGURE VI. 13



Death Curves of S. marcescens In Aerobic Column

FIGURE VI. 14

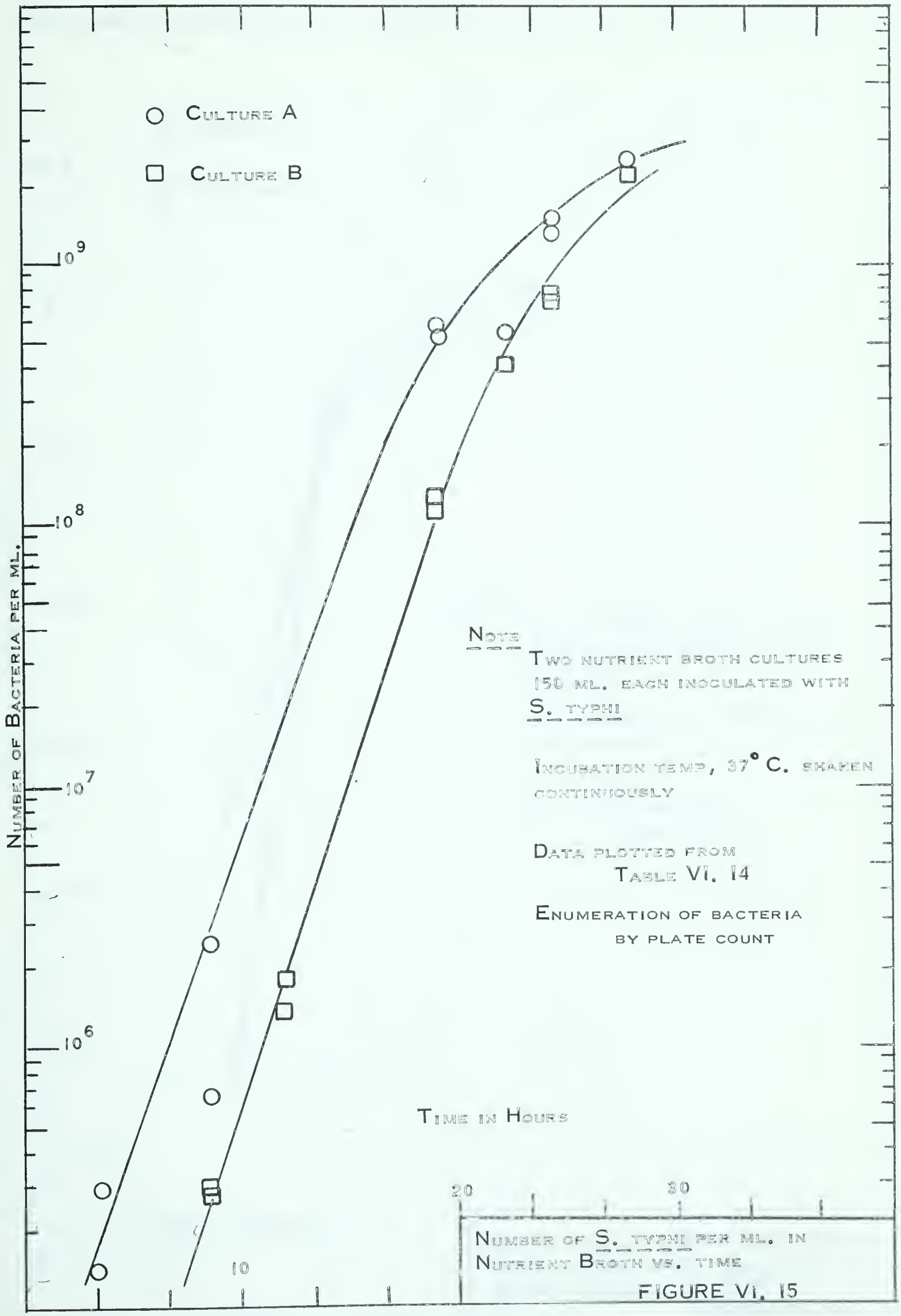
TABLE VI.14

SALMONELLA TYPHI , GROWTH CURVE AND O.D.[1] NUMBERS

TIME HRS.	CULTURE A #/ml. B.S.*	CULTURE A #/ml. N.A.P.°	CULTURE A O.D.	CULTURE B #/ml. B.S.	CULTURE B #/ml. N.A.P.	CULTURE B O.D.
1.75	1.4×10^5	2.9×10^3	0	1.1×10^4	8×10^3	0
4.3	6.5×10^5	2.5×10^6	0	2.8×10^5	2.9×10^5	0
6.0	3×10^6	3×10^6	0	1.8×10^6	1.4×10^5	0
9.6	5.9×10^8	5.6×10^8	.38	1.3×10^8	1.2×10^8	.20
11.0	3×10^7	5.7×10^8	.44	-	4.2×10^8	.33
12.2	1.27×10^9	1.47×10^9	.52	7.2×10^8	7.1×10^8	.45
14.0	-	2.6×10^9	.62	3×10^8	2.3×10^9	.64
23.7	-	2.7×10^9	.69	-	4.5×10^9	.71

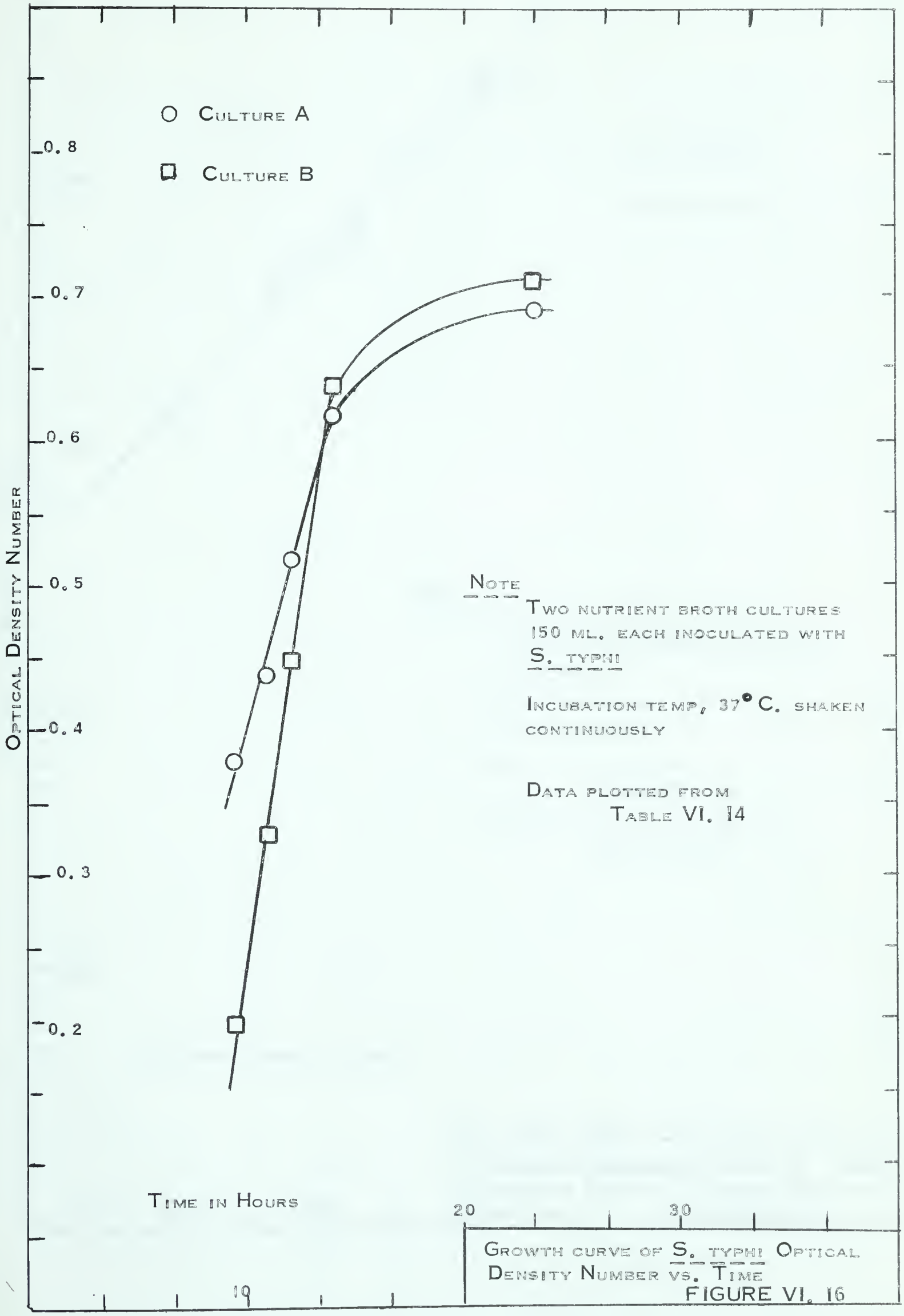
* B.S. - Bismuth Sulphite Agar Plate
o N.A.P. - Nutrient Agar Plate
1 O.D. - Optical Density Number

All analyses made according to (29).
Cultures inoculated Oct. 27, 1964.
Incubated at 37°C in nutrient broth, shaken continuously.
Observations plotted in FIGURES VI.15, 16 & 17.



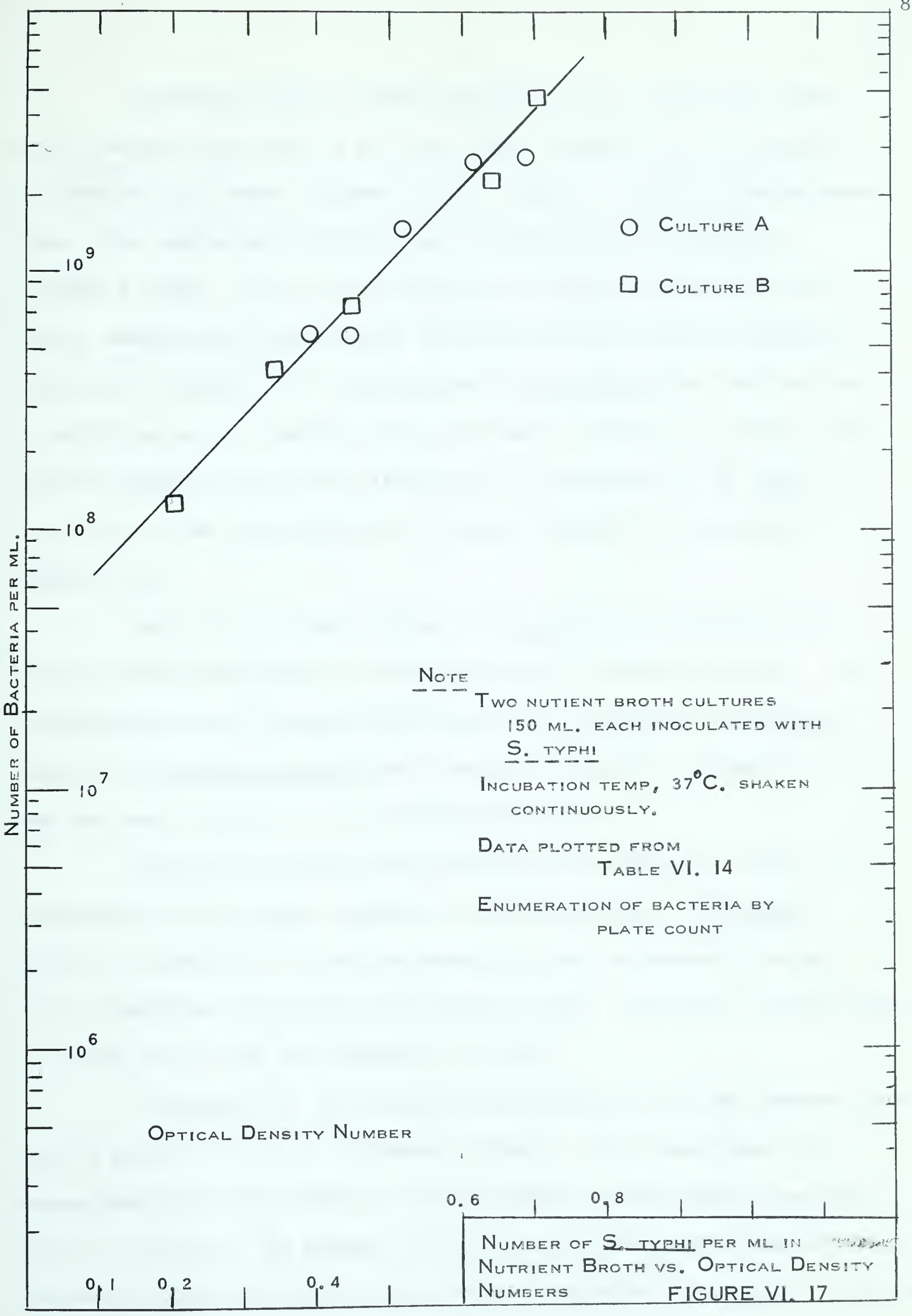
Number of S. typhi per ML. in Nutrient Broth vs. time

FIGURE VI. 15



Growth curve of S. typhi Optical Density Number vs. Time

FIGURE VI. 16



Number of S. typhi per ml in Nutrient Broth vs. Optical Density Numbers

FIGURE VI. 17

Preliminary sets of death curves were run in distilled water, aerobic sewage from column A and river water obtained from the intakes of the Edmonton City Water Treatment Plant located in the North Saskatchewan River. The samples were obtained and inoculated with S. typhi on November 4, 1964. Inoculations effecting 10^7 bacteria per ml. in the 100 ml. samples were made through the optical density method previously described in CHAPTER VI.2. Enumeration of S. typhi per ml. was carried out with the use of standard plating and serial dilution techniques (29). Bismuth Sulphite proved very effective in the isolation of S. typhi from the samples. The resulting data is given in TABLE VI.15 and plotted in FIGURE VI.18.

Tests for the death curves of S. typhi in the anaerobic and aerobic columns commenced on November 13 and 24, 1964 respectively. The testing methods used closely approximated those during the investigation related to Serratia marcescens with one major exception: Bismuth Sulphite agar was used in place of the methylene blue agar.

Safety precautions were taken to guard against the danger of contamination by S. typhi elsewhere in the laboratory. As S. typhi can be air borne it was found necessary to cover the anaerobic columns with polyethylene throughout the testing period. The results are described by TABLES VI.16 to 20 and FIGURES VI.19 to 24.

On December 3, 1964 samples were extracted from the anaerobic and aerobic ponds of the city of Camrose, Alberta. These were taken from approximately the same locations as the original column samples but at a depth of two feet. The purpose of this further investigation was to check the results from the columns and to observe the effect of temperature on the

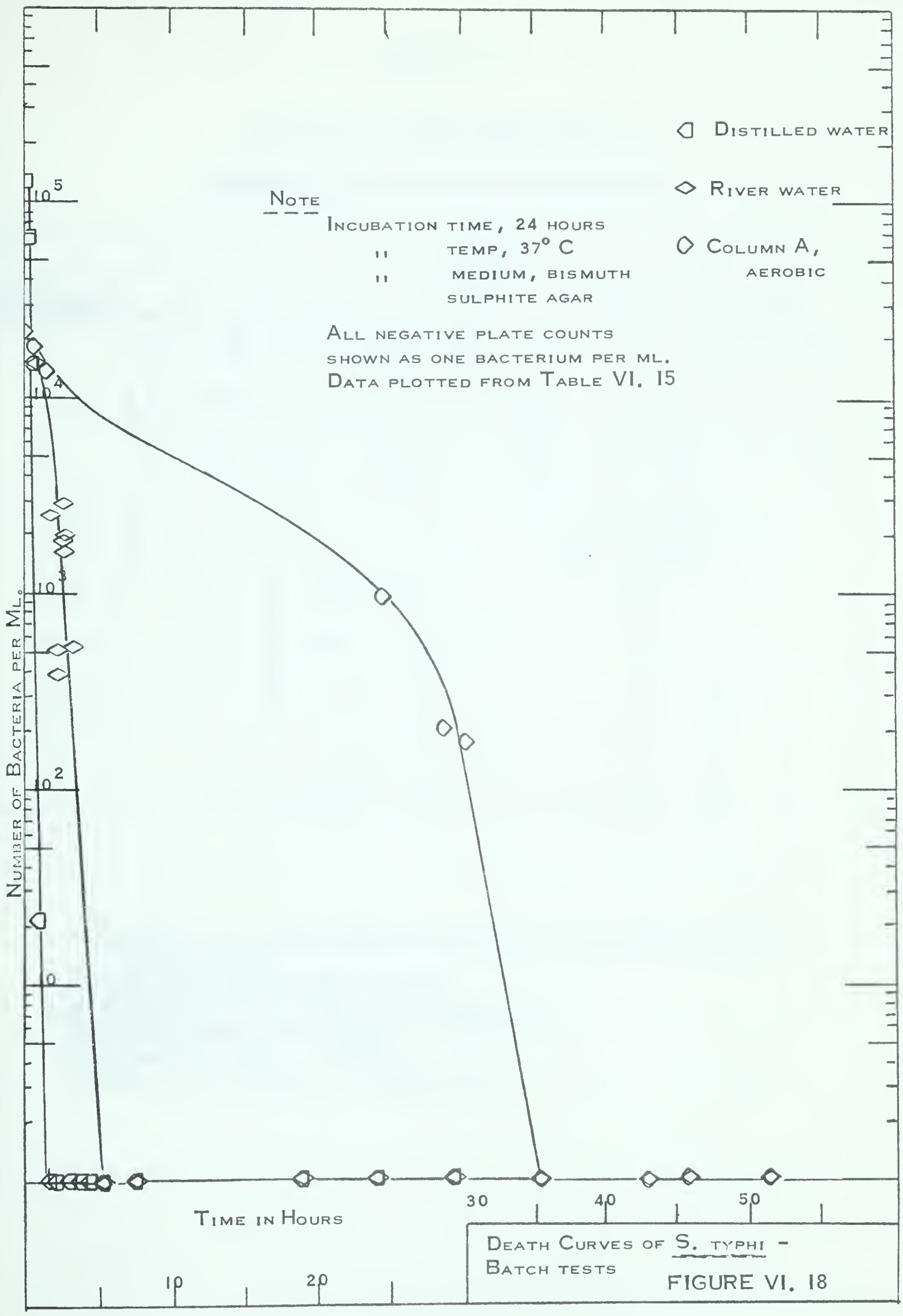
TABLE VI.15

PRELIMINARY TEST DATA FOR THE DEATH CURVES OF SALMONELLA TYPHI IN DISTILLED WATER, AEROBIC COLUMN AND RIVER WATER SAMPLES

DISTILLED H_2O		RIVER WATER*		AEROBIC COL.A	
#/ml.	time-hrs.	#/ml.	Time-hrs.	#/ml.	time-hrs.
1.2×10^5	.2	1.46×10^4	.33	2.2×10^4	0.1
6×10^4	.33	2.4×10^3	.6	1.9×10^4	0.8
2×10^1	.5	3.9×10^3	.6	1.9×10^4	0.8
0	1.1	4×10^3	.6	1.8×10^4	1.3
0	1.2	1.6×10^3	1.2	9.0×10^2	23.9
0	1.3	2.8×10^3	1.2	$2. \times 10^2$	28.1
0	2.1	1.8×10^3	1.8	1.8×10^2	30.6
0	3.1	1.9×10^3	1.8	0	35.6
0	5.1	3.8×10^2	2.2	0	45.2
0	7.5	5×10^2	2.2	0	51.7
0	18.4	5×10^2	3.1		
0	23.8	0	5.1		
0	28.0	0	7.5		
		0	18.4		
		0	23.8		
		0	28.0		
		0	35.5		
		0	45.0		
		0	51.7		

All analyses performed according to (29).
No indication of the presence of Styphi prior to tests.
Tests started Nov. 4, 1964. Held at room temperature approx. 23°C, continuously shaken in 100 ml. portions.

* Taken from the North Saskatchewan River, Treatment Plant intake, Nov. 1964
Observations plotted in FIGURE VI.18.



Death Curves of S. typhi - Batch tests

FIGURE VI. 18

TABLE VI.16

TEST DATA FOR THE DEATH CURVES OF

SALMONELLA TYPHI IN AN AEROBIC COLUMN A

	DEPTH				
TIME-DAYS	6"	2'	4'	6'	Avg.
0	2.1×10^5	2.0×10^5	3.7×10^5	-	2.6×10^5
0.32	1.0×10^5	1.8×10^5	9.8×10^4	1.5×10^5	1.3×10^5
0.5	8.1×10^4	5.2×10^4	3.7×10^4	1.6×10^4	4.7×10^4
1.08	1.7×10^3	2.9×10^3	2.7×10^3	5.2×10^3	4.1×10^3
2.04	0	0	0	9×10^{2}*	0
3.09	0	10^2 *	0	0	0
3.99	0	30 *	0	0	0
8.35	0	0	0	0	0
11.0	0	0	0	0	0
12.26	0	2 *	0	0	0

* Statistically insignificant but presence indicated, omitted from average

All analyses according to (29).

No indication of S. typhi prior to test

Bacterial Enumeration given as #/ml.

Tests started Nov. 13, 1964

Observations plotted in FIGURE VI.19.

TABLE VI.17

TEST DATA FOR THE DEATH CURVES OF

SALMONELLA TYPHI IN ANAEROBIC COLUMN B

	DEPTH				
TIME-DAYS	3"	2'	4'	6'	Avg.
0	2.5×10^5	1.6×10^5	2.5×10^5	-	2.2×10^5
0.31	5.1×10^4	1.3×10^4	1.1×10^4	-	2.5×10^4
0.5	8.0×10^4	8.1×10^4	2.4×10^5	-	1.3×10^5
1.08	-	4×10^4	6×10^4	2.7×10^5	1.2×10^5
2.08	1.1×10^4	2.4×10^4	4.6×10^4	7×10^3	2.2×10^4
3.06	0	6.1×10^3	6.9×10^3	0	3.3×10^3
3.98	2.9×10^3	2.4×10^3	2.3×10^3	8×10^2	2.1×10^3
6.16	0	0	0	0	0
8.33	-	0	0	0	0
10.98	-	0	0	0	0
12.25	-	0	0	0	0

All analyses performed according to (29).
No S. typhi indicated before test
Bacterial enumeration given as #/ml.
Tests started Nov. 13, 1964
Observations plotted in FIGURE VI.20.

TABLE VI.18

TEST DATA FOR THE DEATH CURVES OF

SALMONELLA TYPHI IN ANAEROBIC COLUMN C

	DEPTH				
TIME-DAYS	6"	2'	4'	6'	Avg.
0	1.4×10^5	2.8×10^5	-	-	2.1×10^5
0.31	2.3×10^5	1.5×10^5	1.4×10^5	-	1.7×10^5
0.5	1.4×10^5	1.6×10^5	9.6×10^4	1.1×10^5	1.3×10^5
1.08	1.1×10^4	6.6×10^4	6.8×10^4	1.2×10^5	0.7×10^5
2.08	7.5×10^4	7.0×10^4	9.0×10^4	9.6×10^4	8.3×10^4
3.02	4.8×10^4	3.8×10^4	-	6×10^4	4.9×10^4
3.98	2.1×10^4	1.9×10^4	1.9×10^4	3.1×10^4	2.3×10^4
6.16	0	4×10^3	0	0	1.0×10^3
8.33	5.2×10^2	4.3×10^2	5.5×10^2	4.0×10^2	4.8×10^2
10.98	40*	30*	50*	0	0
12.25	0	0	0	0	0

* Statistically insignificant although indicative of presence of S.typhi, omitted from averages
All analyses performed according to (29).
No evidence of S. typhi observed before test
Bacterial Enumeration given as #/ml.
Tests started Nov. 13, 1964
Observations plotted in FIGURE VI.21.

TABLE VI.19

TEST DATA FOR THE DEATH CURVES OF

SALMONELLA TYPHI IN ANAEROBIC COLUMN D

	DEPTH				
TIME-DAYS	3"	2'	5'	6'	Avg.
0	2.1×10^5	1.8×10^5	2.0×10^5	-	2.0×10^5
0.31	1.2×10^5	7.4×10^4	-	-	0.9×10^5
0.5	1.1×10^5	5.7×10^4	6.9×10^4	2.9×10^5	1.3×10^5
1.08	4.7×10^4	6.0×10^4	3.7×10^4	1.6×10^5	7.5×10^4
2.08	1.9×10^4	1.6×10^4	2.6×10^4	4.7×10^4	2.7×10^4
3.06	1.2×10^4*	1.5×10^4	1.1×10^4	2.2×10^4	4.8×10^4
3.98	6.9×10^3	7.1×10^3	5.6×10^3	1.2×10^3	5.2×10^3
6.16	1.7×10^3	2.5×10^3	60*	5.8×10^3	3.3×10^3
8.33	85*	1.1×10^3	7.9×10^2	6.7×10^2	8.7×10^2
10.98	130*	0	0	130*	0
12.25	0	0	0	0	0

* Statistically insignificant but indicates trace count, omitted from average.

All analyses performed according to (29).

No evidence of Styphi was indicated before test

Bacterial Enumeration given as #/ml.

Tests started Nov. 13, 1964.

Observations plotted in FIGURE VI.22.

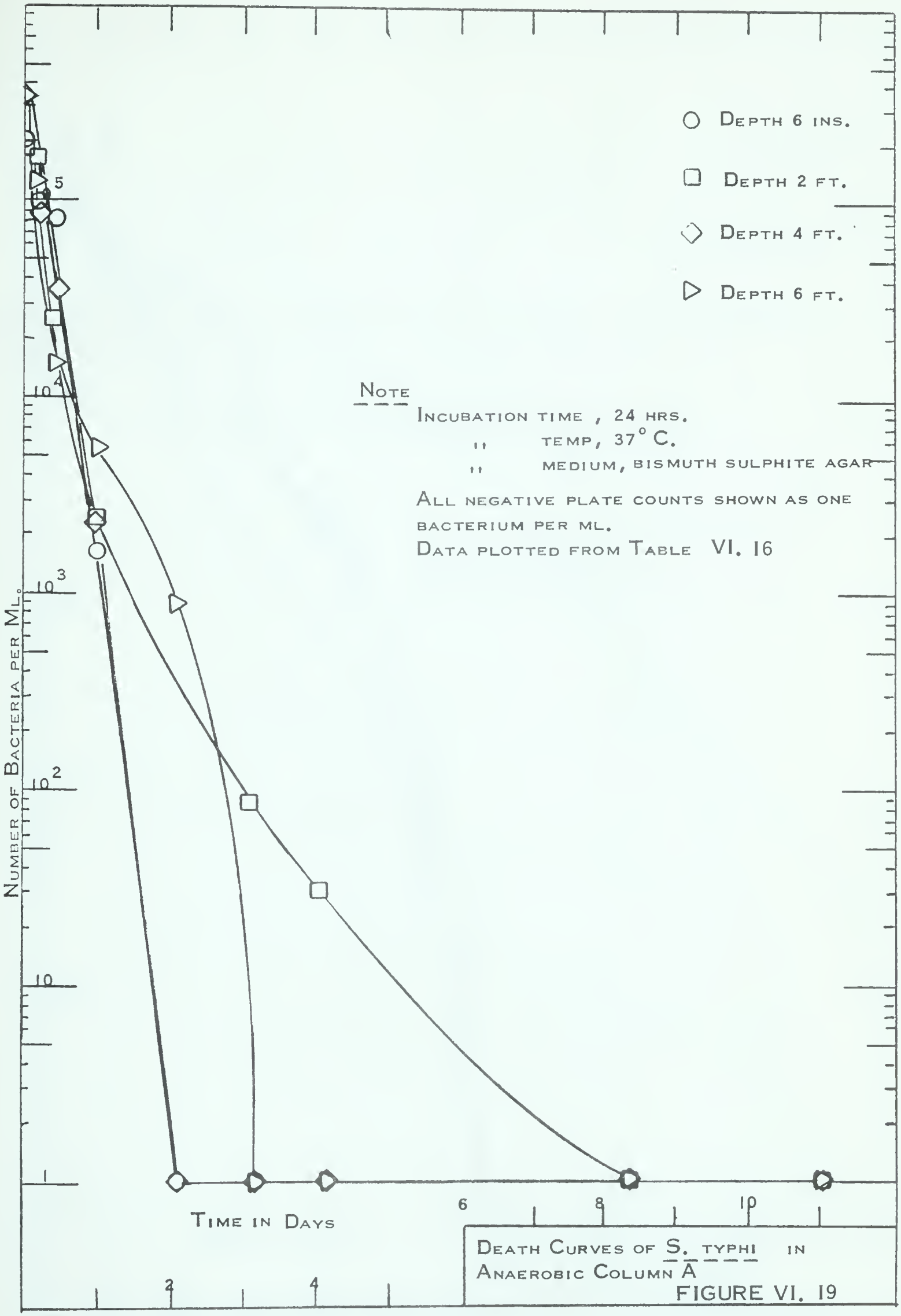
TABLE VI.20

TEST DATA FOR THE DEATH CURVES OF SALMONELLA TYPHI

IN AEROBIC COLUMN

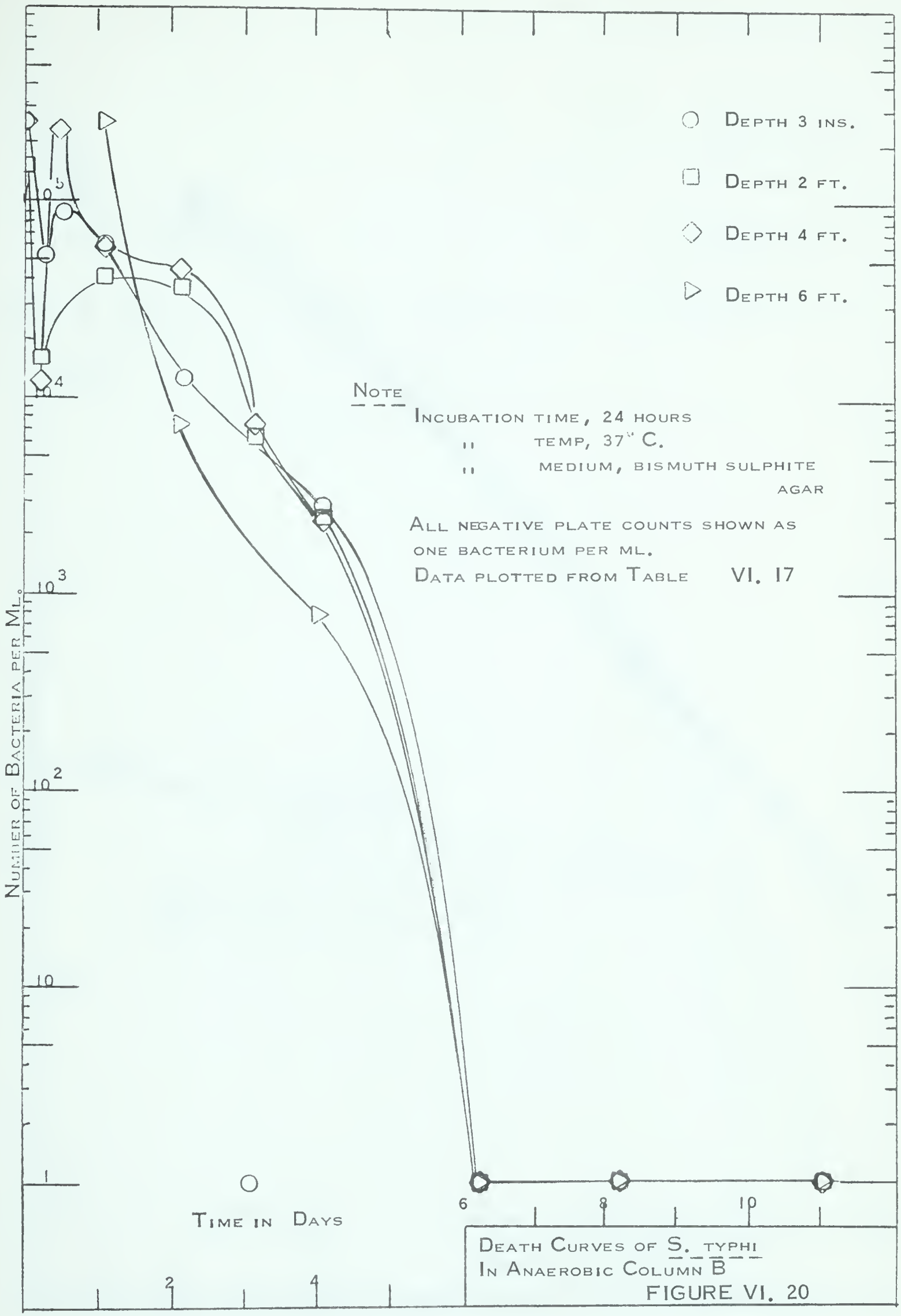
TIME-HOURS	DEPTH		
	3"	2'	3' - 6"
0	5.8×10^5	2.3×10^5	1.8×10^5
6.6	2.5×10^4	1.9×10^4	1.7×10^4
24.5	3.0×10^2	2.2×10^2 *	3.0×10^2
31	0	30*	30*

* Statistically insignificant but indicates trace count, omitted from average.
All analyses performed according to (29).
No evidence of S. typhi before test.
Bacterial enumeration given as # per ml.
Test started November 24, 1964.
Observations plotted on FIGURE VI.14.



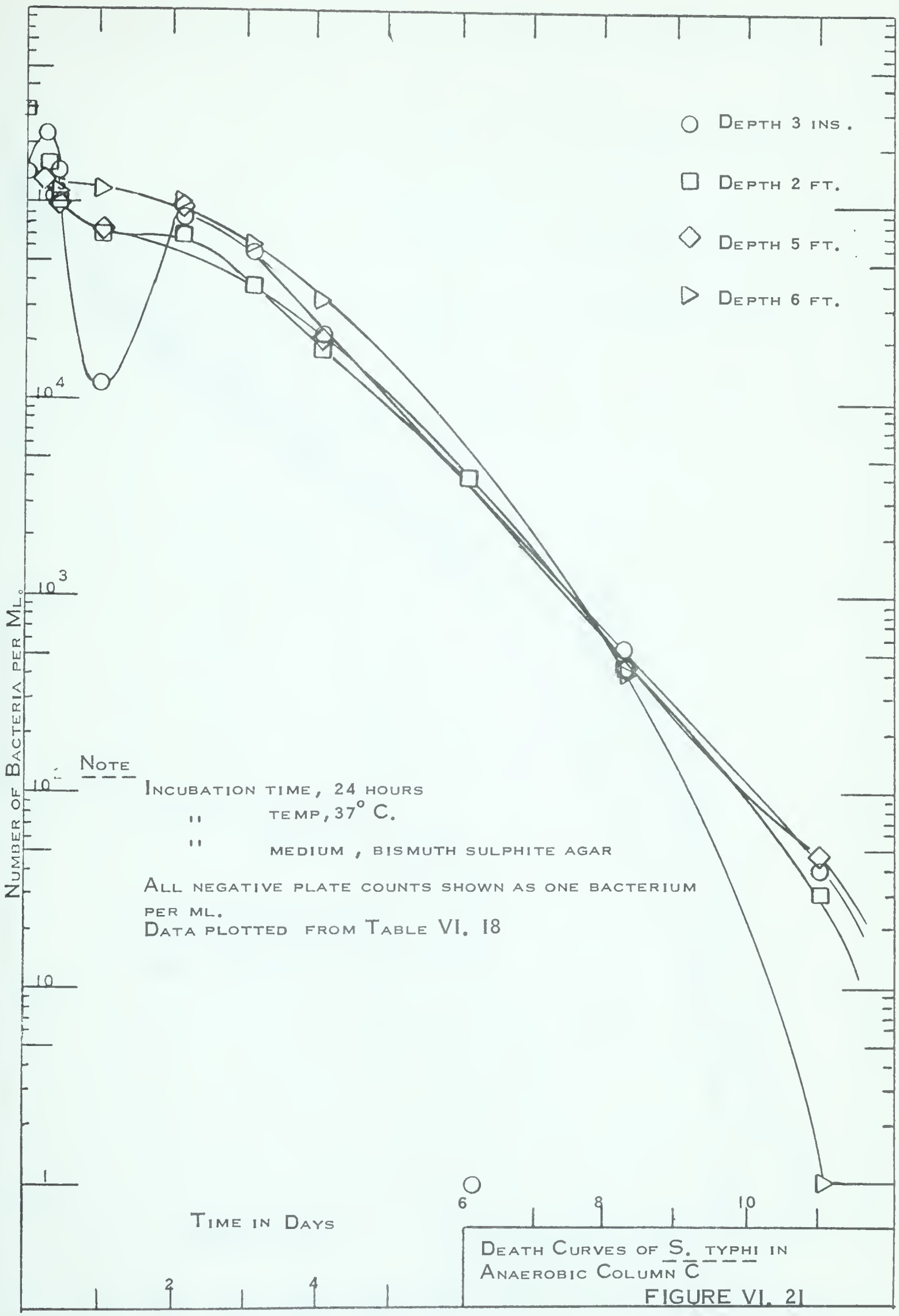
Death Curves of S. typhi in Anaerobic Column A

FIGURE VI. 19



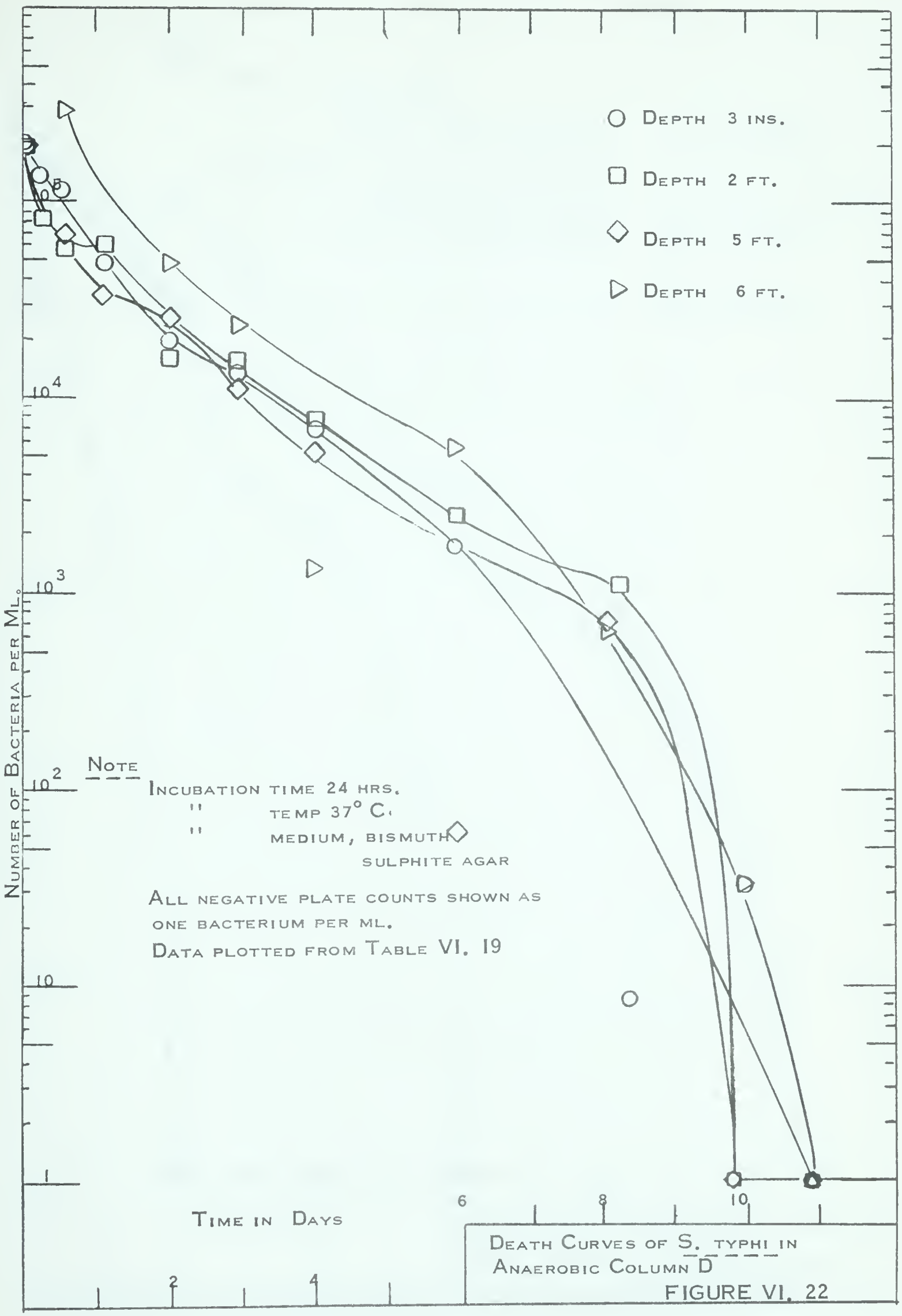
Death Curves of S. typhi
In Anaerobic Column B

FIGURE VI. 20



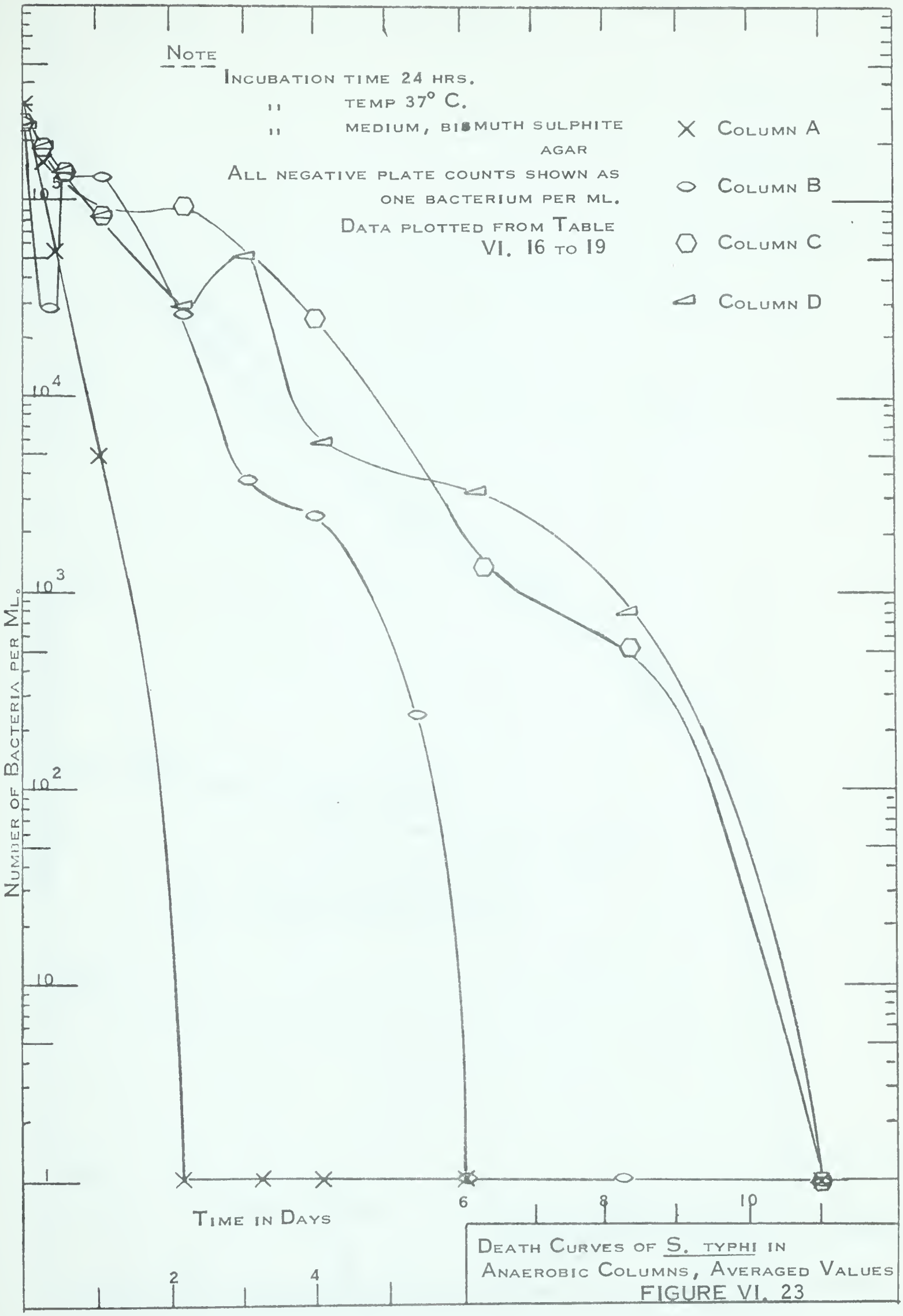
Death Curves of S. typhi in Anaerobic Column C

FIGURE VI. 21



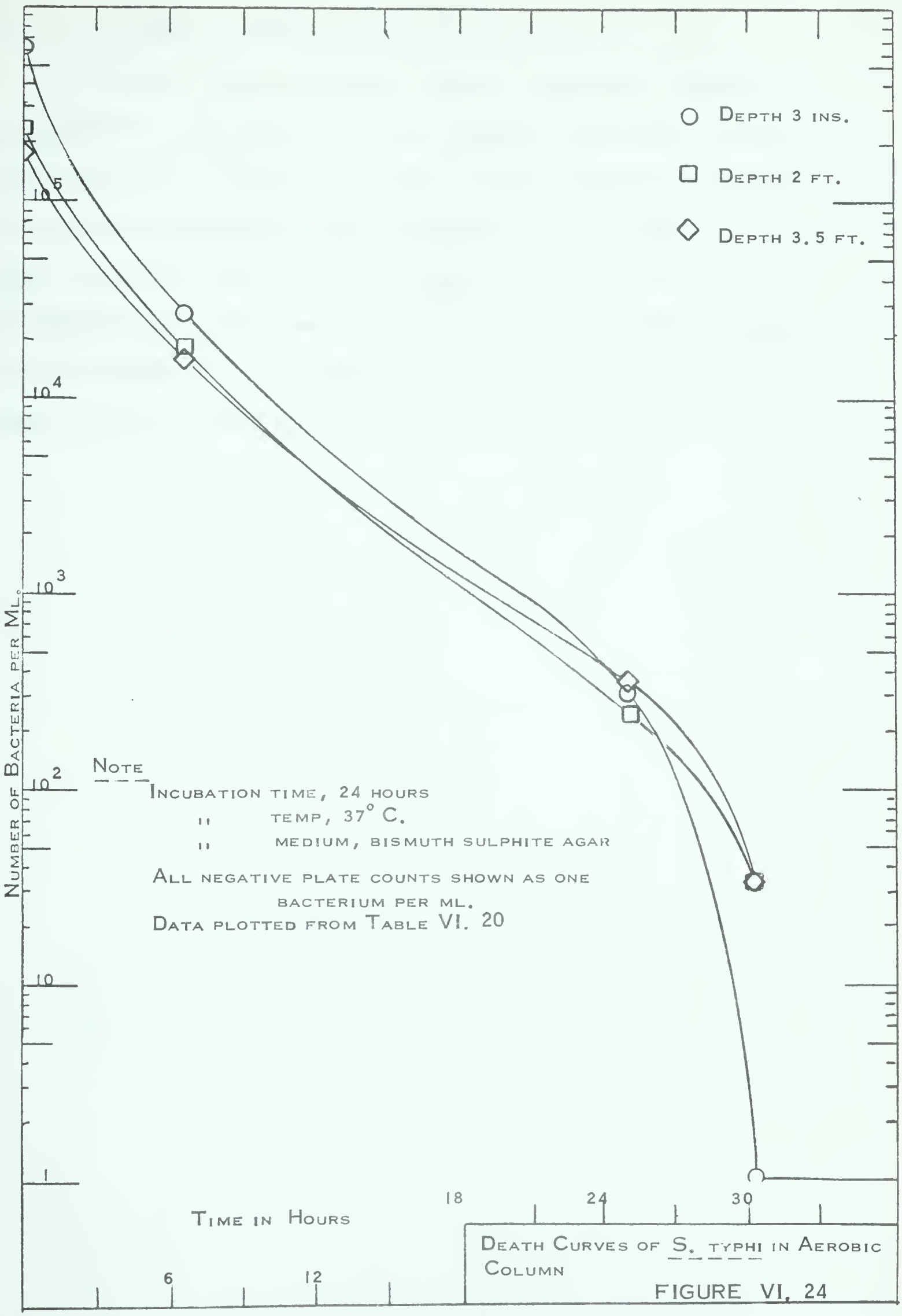
Death Curves of S. typhi in Anaerobic Column D

FIGURE VI. 22



Death Curves of S. typhi in Anaerobic Columns, Averaged Values

FIGURE VI. 23



Death Curves of S. typhi in Aerobic Column

FIGURE VI. 24

longevity of S. typhi in sewage oxidation ponds.

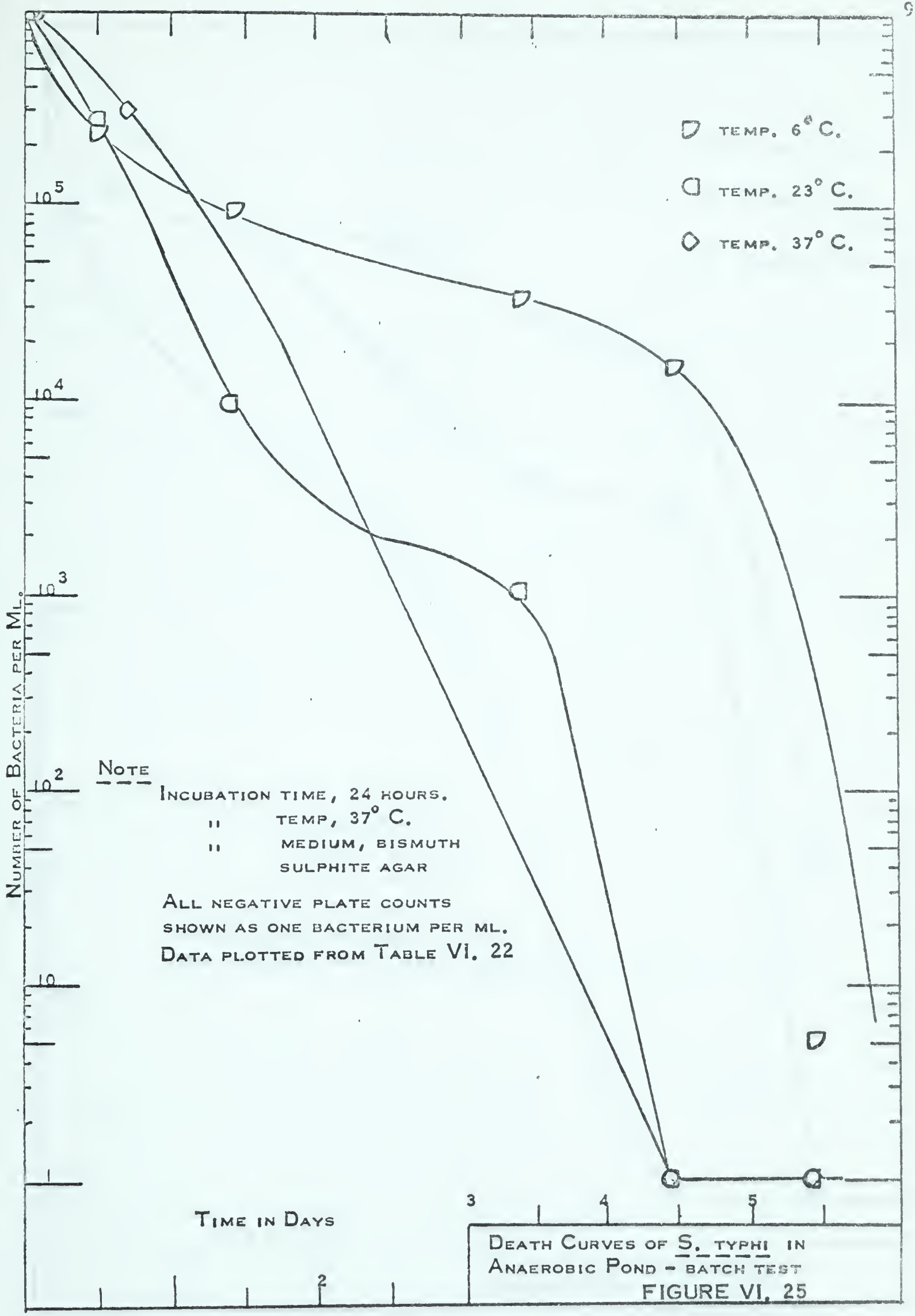
The samples were inoculated to effect a bacterial population of 10^6 S. typhi per ml. of sewage. 100 ml. volumes of each sample were set up at temperatures of 6°. 23° and 37°C. The continual shaking by mechanical shakers was interrupted only to test the samples for the enumeration for S. typhi. The medium used to isolate S. typhi from sewage organisms was Bismuth Sulphite agar. The observations are reported in TABLE VI.22 and described by FIGURES 25 & 26. Chemical analysis of the pond waters was performed, the results being tabulated in TABLES VI.1, 2, 8 & 9.

TABLE VI.21

BATCH TEST DATA FOR DEATH CURVES OF SALMONELLA TYPHI IN CAMROSE PONDS' SEWAGE

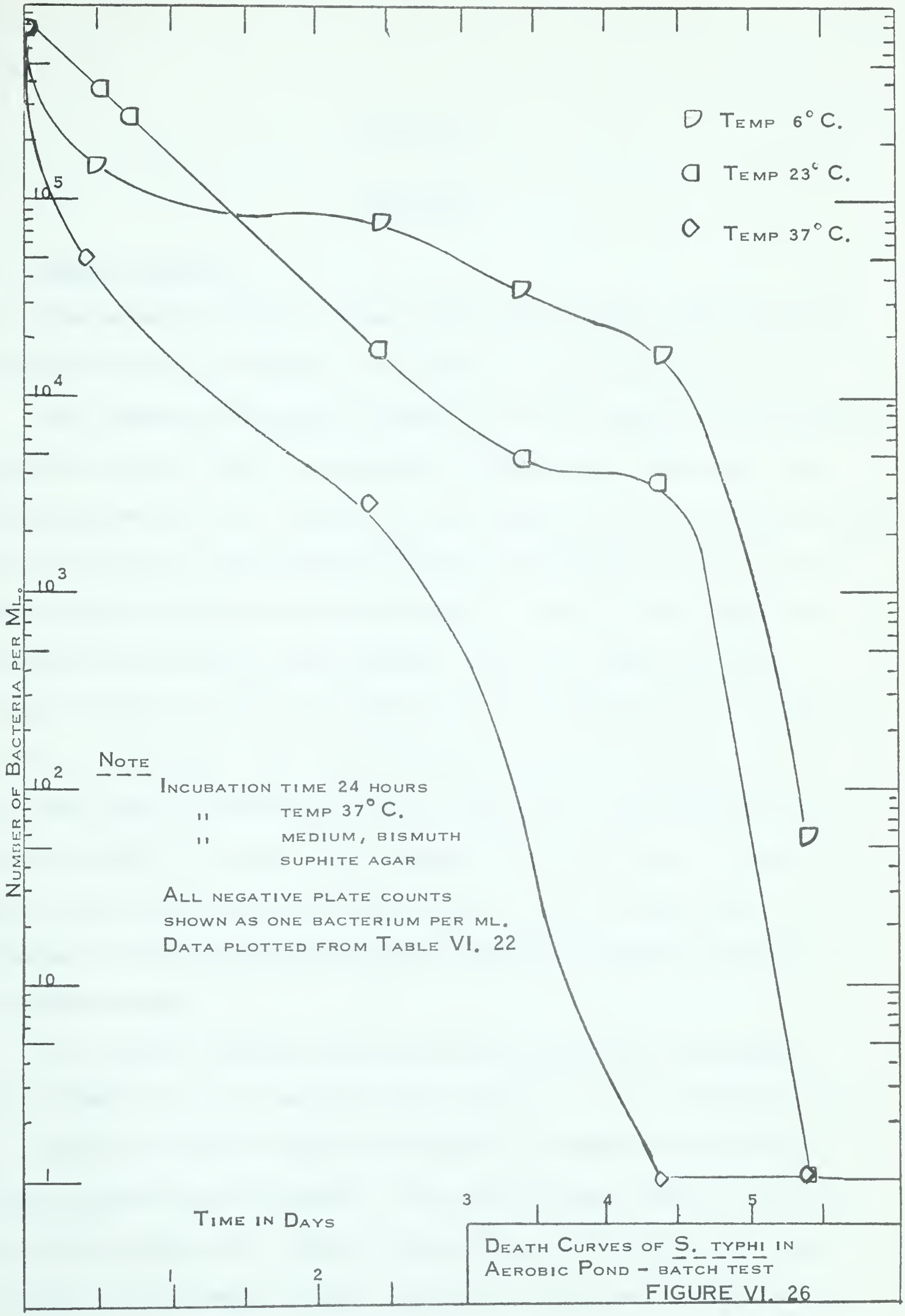
TEMPERATURE	6°C		23°C		37°C	
POND	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC
TIME-DAYS						
0	6.8×10^5	1.1×10^6	6.8×10^5	1.1×10^6	6.8×10^5	1.1×10^6
0.5	1.4×10^5	2.3×10^5	3.5×10^5	2.6×10^5	4.8×10^4	-
0.7	-	-	2.4×10^5	2.8×10^5	2.4×10^5	2.9×10^5
2.4	7.2×10^4	9.3×10^4	1.6×10^4	8^* x 10^3	2.4×10^3	-
3.4	3.3×10^4	3.3×10^4	4.2×10^3	1^* x 10^3	-	-
4.4	1.6×10^4	1.4×10^4	3.3×10^3	0	0	0
5.4	56	5^*	0	0	0	0
6.4	8^*	5^*	0	0	0	0

* Statistically insignificant, however indicated presence of bacteria, omitted from average.
All analyses performed according to 29 .
No evidence of S. typhi in blanks before testing.
Bacterial enumeration given as #/ml.
Sampled December 3, 1964. Depth 2'.
Observations plotted in FIGURES VI.25 & 26.



DEATH CURVES OF S. TYPHI IN ANAEROBIC POND - BATCH TEST

FIGURE VI. 25



Death Curves of S. typhi in Aerobic Pond - batch test

FIGURE VI. 26

CHAPTER VII

DISCUSSION

VII.1 Chemical Analyses

The chemical analyses of the aerobic and anaerobic ponds revealed the decided effect that depth has on any pond's chemical characteristic.

The incoming raw sewage's effect is clearly marked by the low B.O.D. rating at the one foot depth as described by FIGURE VI.3. Below two feet the B.O.D. continues to rise rapidly as the sludge on the bottom is approached. Through the activity of the methane producers sludge particles often become attached to gas which raises them to the surface. The surface waters are often filled with particles such as these. Often the methare producers will be so active as to cause a boiling effect where huge masses of sludge will rise to the surface. The result of this is shown in higher B.O.D.'s at the surface than at depths about the two foot level. The alkalinity and pH curves in FIGURE VI.2 show little variance in pH with depth. Between the surface and seven foot level a pH range of only 7.3 to 6.7 was found. The bicarbonate, in this case, total alkalinity dropped slightly as did the pH with increasing depth.

The effect of algae on the aerobic pond was clearly evidenced as shown in FIGURES VI.4 & 5. As mentioned in Chapter I.2, the surficial algae act as a protective barrier against the high solar radiation in protecting the algae at depths below six inches. The most profound effect is noted in the pH curve of FIGURE VI.5. Algae receive CO_2 from the sewage waters and in its place release oxygen causing a rise of pH. The rise is noted to be

maximum around the depth of one foot. The normal and bicarbonate alkalinities are also greatly affected by this increasing pH as may be seen by FIGURE VI.4.

The chemical analyses of the samples of the Camrose ponds taken on December 3, 1964 revealed how little effect the temperature has on the pH and alkalinities. Although the temperature dropped from the July 22 values of around 23°C to the winter temperatures of approximately 6°C, the anaerobic pH onlychanged from 7.1 to 7.8, and the aerobic from 9.0 to 8.3. The alkalinities changed only slightly as shown in TABLES VI.1 & 2.

A wide variance of pH may be in the aerobic pond as it is dependent upon algal action which fluctuates over the day and night. Ice cover also effects algal activity.

A comparison between the ponds and columns may be made through chemical analyses as shown in TABLES VI.1 to 7. Due to the differing loading conditions in the columns the chemical characteristics observed therein remained only within a wide range of the original characteristics of July 22, 1964.

The pH levels of the anaerobic columns remained between 6.7 and 8.3 during the testing period which are comparable to the pH's of the pond on July 22 (6.8 to 7.3) and December 3 (6.8 to 7.3). The pH's of the aerobic column remained between 7.3 and 9.6, while in the aerobic pond on July 22 they were observed to be between 8.7 and 9.2, and on December 3, 8.3. The effect of algae in both the aerobic column and pond was noted through the relatively high pH's observed therein.

The alkalinities of the anaerobic were observed to be all bicarbonate in nature as in the pond. These ranged in the column during the testing period between 880 and 1672 ppm. as $CaCO_3$. An increase in alkalinities was observed over the testing period. The pond alkalinities on July 22 were

290 - 560 ppm. as $CaCO_3$ and on December 3, 589 ppm. as $CaCO_3$. The alkalinities of the aerobic column remained between 20 and 88 ppm. as $CaCO_3$ normal carbonate and 212 and 328 ppm. as $CaCO_3$ bicarbonate. The ponds were originally analyzed on July 22 to contain between 60 and 300 ppm. as $CaCO_3$ normal carbonate and between 80 and 455 ppm. as $CaCO_3$ bicarbonate. The observed total alkalinity in the aerobic pond on December 3, 1964 was 730 ppm. as $CaCO_3$ bicarbonate.

The dissolved oxygen of the anaerobic column remained zero throughout the testing program as was the dissolved oxygen in the anaerobic pond on July 22. The aerobic pond required additional oxygen to keep up the dissolved oxygen content. Air was bubbled through the sewage for this purpose as described in CHAPTER VI.1. As shown in TABLE VI.3 saturation levels of D.O. were sometimes exceeded indicating the effectiveness of algae in producing dissolved oxygen above saturation levels. Algae was present in such quantities as to be easily seen in the column.

VII.2 Discussion of Tests Related to Serratia marcescens

Originally the plate counts of S. marcescens were to be made through the use of the millipore filter and the field monitor. It was concluded by the author following many trial runs with Serratia marcescens on the millipore filter, that this method resulted in an erratic count. The greatest source of error was caused by the small area available for each colony, through bacterial competition. This resulted in a highly crowded and competitive plate which often resulted in the Serratia marcescens being covered by other colonies before 24 hours of incubation at 37°C had elapsed. Often the sample being filtered would bypass the filter itself through the supporting pad. This was the fault of the monitor. No tests were carried out using

the standard millipore techniques (1) without using the field monitor method.

The purpose of obtaining a growth curve and corresponding density numbers for Serratia marcescens in tryptocase soy broth was to estimate the number of Serratia marcescens per ml. in suspension in a medium prior to the inoculation of the columns. A large number of 10 ml. volumes of the broth were inoculated in order to obtain the optical density numbers of many cultures. The resulting variation of O.D. as shown by TABLE VI.9 and FIGURE VI.8 is a result of using such a procedure.

The columns were inoculated with S. marcescens as described in Chapter III.1. The standard serial dilution and plate count techniques (29) were used and the resulting death curves are in FIGURES VI.9 to 14. For plotting purposes negative plate counts or those upon which Serratia marcescens were not observed have been shown as one bacteria per ml. It is noted that there is no essential difference in the death pattern between the depth of any one column. In contrast to this there was a distinct difference between the aerobic and anaerobic death rate. In the aerobic column Serratia marcescens originally had a population density of over 10^3 per ml. which declined within 12 hours to zero bacilli per ml. Even under the differing loading conditions essentially no differences in the death pattern was observed between the anaerobic columns. The average values of the number of Serratia marcescens per ml. at any given time over the length of any one anaerobic column were used in plotting FIGURE VI.13. Some plate counts were plotted in the FIGURES VI.9 to 12 to show traces of the bacilli when the numbers of colonies per plate were less than 30, these are distinguished on TABLES 10, 11 & 13 by the use of asterisks. These values have been omitted in calculating the average values due to their having little statistical significance. In nearly all cases the bacterial population of Serratia marcescens remained in between

10^3 and 20^4 per ml. for a period of 24 hours and then began a rapid decline to 0 over the next 6 hours. No traces of Serratia marcescens were found during the ensuing studies of sewage in the columns.

VII.3 Discussion of Tests Related to Salmonella typhi

FIGURES VI.15, 16 & 17 describe the growth rate of S. typhi in nutrient broth and the corresponding optical density numbers. The procedures used in obtaining these were similar to those used with S. marcescens with one exception: only two cultures, each 100 ml. were inoculated with S. typhi in order to more closely define the growth curve. Bismuth Sulphite agar and nutrient agar were used in enumerating the S. typhi per ml. for purposes of comparison between the media. Bismuth Sulphite proved very efficient in the isolation of S. typhi, however care in the preparation and use of the medium must be taken.

As stated previously in CHAPTER VI.2 the optical density numbers were used in order to obtain a straight line graph when the log of the number of S. typhi per ml. is plotted against the optical density number. FIGURE VI.17 shows such a plot and the resulting straight line graph. Optical density numbers were observed for populations between 10^8 and 10^{10} bacilli per ml. Frobisher (13) describes the accelerated growth phase of many bacterial species following a straight line when the logarithm of the number of bacteria per ml. is plotted against time. FIGURE VI.15 shows such a plot and the resulting straight line graph. In view of this, a plot of optical density numbers versus time also results in a straight line graph. The end of the log growth phase is marked when the plot varies from the straight line. At this point bacterial death begins as does the phase of negative growth acceleration.

Bismuth Sulphite agar compared very favorably with the nutrient agar plates in the enumeration of S. typhi per ml. The results of the batch tests performed through the inoculation of S. typhi into 100 ml. samples of distilled water, river water, and sewage obtained from the aerobic column are shown in FIGURE VI.18. There is a decided difference in the longevity between the three samples. Death occurred within 1.1, 5.1 and 35.6 hours in the distilled water, river water and column sewage respectively.

As was the case of Serratia marcescens negative plate counts are shown as one bacterium per ml. for plotting purposes on all death curves. Death curves in the columns of S. typhi are shown in FIGURES VI.19 to 24. There is a distinct difference between the anaerobic columns. The aerobic death curves serve to accentuate this difference.

The columns were inoculated with S. typhi resulting in populations of 10^5 to 10^6 of the typhoid bacilli per ml. of sewage. Death of these bacilli occurred in the anaerobic columns A, B, C and D within 2.04, 6.16, 10.98 and 8.33 days respectively. Traces of S. typhi were found in column A after 12.26 days had elapsed and in columns C and D after 10.98 days. However, these are not considered of any statistical significance, as such traces might well have been caused through the contamination of plates.

On December 3, 1964 samples were extracted at a depth of 2 feet from the Camrose ponds in similar locations as the original columns were sampled. These were taken back to the laboratory and following a chemical analyses 100 ml. portions of aerobic and anaerobic sewage were inoculated with S. typhi and set on constant stirring apparati at temperatures of 6°. 23° and 37°C. Samples were extracted for the enumeration of S. typhi on Bismuth Sulphite agar at various times throughout the testing procedure. The resulting death curve data is tabulated in TABLE VI.21 and plotted in FIGURES VI.25 & 26.

The marked effect of temperature is shown by the fact that S. typhi at higher temperatures was unable to attain as high populations and died more rapidly.

There was little difference in death curves between samples of aerobic and anaerobic origin at similar temperatures. Possibly this was the result of a non-representative sample being taken from the pond on December 3.

The sewage used as nutrient was sterilized to avoid the addition of any other organism which was not natural to oxidation ponds. The populations of bacteria were kept up through the continual addition of nutrient. Throughout the S. marcescens testing program a constant population approximating 10^5 bacteria per ml. of facultative and obligate aerobes were evidenced.

It was assumed throughout the testing period that the microbiological conditions in the columns would be representative of those in the oxidation ponds. As a check, an enumeration of the aerobic obligate and facultative anaerobic bacteria was carried out following the tests with S. typhi. In all columns the number of these bacteria was observed to be within 10^5 to 10^6 per ml. of sewage. These populations are similar to those observed in the ponds (9).

CHAPTER VIII

CONCLUSIONS

Through the chemical analyses of the oxidation ponds it was shown that the conditions in a pond are by no means homogeneous throughout, despite the effects of mixing by influent sewage, wind action and turnover.

The chemical and microbiological conditions tested in the ponds were observed to be within the range of conditions tested in the columns throughout the investigation. As the columns were fed at different loading rates the conditions within the columns were representative of ponds under similar loading rates.

The method of estimating the population of bacteria in suspension through the use of optical density numbers proved entirely successful for the purposes of this investigation. The reason why the resulting scatter was found about the curves on FIGURE VI.8 relating to the growth curve of Serratia marcescens, is that each point is representative of an individual 10 ml. culture. Thus the growth curve is not representative of only one culture but of many. The growth and optical density number curves of Salmonella typhi however, show little scatter. The curves shown in FIGURES VI.15, 16 & 17 represent only two cultures. The success of enumerating population densities with a reasonable amount of accuracy, is also evident in these figures. The accuracy obtained through the use of serial dilution and plating techniques was found to be sufficiently accurate for purposes of this investigation.

Bismuth Sulphite agar as shown by these graphs and figures proved

entirely satisfactory in isolating and enumerating the S. typhi bacillus in sewage. As mentioned in CHAPTER V.8 great care must be taken in the preparation and storage of Bismuth Sulphite agar. The millipore filter technique used in conjunction with the field monitor proved unsatisfactory for the enumeration of Serratia marcescens in sewage. Its use was discontinued in favor of the standard plate counting technique. Use of the millipore filter with pyrex filter holder and vacuum pump was not covered in the tests.

The secondary objective of this investigation was to compare the death rates of Serratia marcescens to S. typhi under similar sewage nutrient conditions. If their death rates had been similar, Serratia marcescens might have been used as a non-pathogenic indicator organism to indicate the longevity of S. typhi in ponds through the inoculation of Serratia marcescens into the ponds. As recorded by FIGURES VI.9 to 14 in comparison with FIGURES VI.19 to 24 the death rates were by no means similar. S. typhi survived in the anaerobic columns for periods of up to 11 days and in the aerobic columns up to only 2 days. On the other hand Serratia marcescens survived over periods of only 28 hours in the anerobic columns and 12 hours in the aerobic. It is concluded that Serratia marcescens may not be used as an indicator organism in the Salmonella typhi longevity testing procedures.

The death curves of Serratia marcescens in the anaerobic columns display a very similar pattern. The normal growth in death curves of bacteria as described by Frobisher (13) include an equilibrium phase called the maximum stationary phase at which an equilibrium population is reached. FIGURES VI.9 to 14 show all death curves reaching this equilibrium population and after maintaining it for an extended period suddenly die off within 3 to 4 hours. Such a rapid die-off is unusual in the death of bacteria. It was observed that the equilibrium populations of S. marcescens in the anaerobic

columns were similar regardless of the differing loading rates of the columns. This suggests that the populations of S. marcescens in the anaerobic columns were independent of nutrient conditions.

Death curves of Salmonella typhi in distilled water, river water and sewage from the surface of the aerobic column were observed. FIGURE VI.18 gives a graphical representation of the results. Death occurred in the distilled water within 1.1 hours, in the river water within 5.1 hours and in the sewage within 35.6 hours. These results indicate that the Salmonella typhi death rate is dependent upon the nutrient conditions. McKinney (26, 27) has proven this fact and also uncovered the nature of the essential metabolite as tryptophan.

Tests on the columns further served to verify the above conclusion that Salmonella typhi survival is dependent upon nutrient availability and bacterial competition. Death of Salmonella typhi occurred within a period of 1.29, 2.04, 6.16 10.98 and 8.33 days in the aerobic, anaerobic A, B, C and D columns respectively. The corresponding column loadings or feeding rates were 50, 500, 2000, 4000 and 6000 pounds B.O.D. per acre per day. These loadings and death rates suggest that the longevity of typhoid in sewage oxidation ponds is proportional to the loading of the pond up to a loading of 4000 pounds B.O.D. per acre per day.

The death curves as shown in FIGURES VI.19 to 26 do not follow a straight line log plot as described by Frobisher (13). Equilibrium populations were not established in the columns as in the case of Serratia marcescens. The death curves reveal a high reduction in numbers immediately following inoculation. After this a reduction in the slope of curve is noted, it is postulated that this reduction in slope is equivalent to the equilibrium phase. However, S. typhi was incapable of reaching or maintaining

an equilibrium population at any time.

There was no major difference or pattern of differences in the death rates of S. typhi at different depths in any one column. A batch test of the pond waters was made to obtain death curves of S. typhi as a check to the foregoing columns and to indicate the effect of temperature on the longevity of S. typhi. FIGURES VI.25 and 26 show the resulting death curves between the aerobic and anaerobic batch samples. This was probably caused by a non-representative sample being taken from the aerobic pond. The shape of curves observed was similar to those found in the anaerobic column testings.

Temperature showed its effect in the S. typhi populations and longevity. The samples maintained at a lower temperature exhibited greater S. typhi densities for a longer survival time. At lower temperatures the typhoid bacillus was not able to reach its near equilibrium phase as quickly nor sustain it at higher levels attained at the higher temperatures. This was due to the metabolism of the bacilli being slowed down by lower temperatures thus making the nutrient available for a longer period of time. The result of availability of nutrient enables the typhoid bacilli to establish a near equilibrium population. Had there been more nutrient available in the sewage the 37°C sample would have established an equilibrium population higher than that of the lower temperatures in accordance with conclusions reached by Bowmer (5). In order to avoid excess mixing, the anaerobic columns were not fed during the time of testing. Thus the inflow of tryptophan was stopped. It is postulated that S. typhi could have survived for a considerably longer period of time than was observed in these columns if trytophan had been supplied. The influent of the aerobic column was not halted, resulting in a more representative sample system.

In view of this the anaerobic pond represents a serious health hazard if there is a typhoid case or carrier in the town whose wastes are being treated by oxidation ponds. This hazard is lessened considerably by the much longer detention period of the anaerobic ponds if they are included in the system.

It is to be noted that the inoculation of S. typhi were made through the use of suspensions of the bacilli in nutrient broth. The longevity of S. typhi in oxidation ponds might be extended if S. typhi entered the pond in a matrix of feces being thusly surrounded by protective coatings of organic matter.

CHAPTER IX

SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

IX.1 Summary of Conclusions

1. The columns used to sample and represent the oxidation ponds were found to be entirely satisfactory for purposes of this investigation. The chemical and microbiological conditions tested in the ponds were within the range of conditions in the columns tested during the program. The columns being fed at different loading rates, were representative of ponds under similar loading rates.

2. Serratia marcescens exhibited death curves which were not similar to those observed for Salmonella typhi. In view of this, Serratia marcescens cannot be used as an indicator organism through its inoculation into ponds to test the longevity of the typhoid bacillus.

3. The death rate of Salmonella typhi in the oxidation pond is dependent upon the loading of that pond. Its death is primarily caused by a lack of nutrient in the oxidation pond.

4. Death of Salmonella typhi occured within periods of 1.29, 2.04, 6.16, 10.98 and 8.33 days in columns with the corresponding loading rates of 50, 500, 2000, 4000 and 6000 pounds B.O.D. per acre of surface area per day. These survival times and loadings suggest that the longevity

of typhoid bacilli in sewage oxidation ponds is proportional to the loading of the ponds up to 4000 pounds of B.O.D. per acre of surface area per day.

5. The death rates of S. typhi inoculated into distilled and river water further verified that the survival of S. typhi is dependent upon nutrient availability.

6. If an oxidation pond system which serves a community in which there is a typhoid case or carrier consisting solely of anaerobic ponds is discharging effluent into river courses or lakes, there is a serious danger that typhoid bacilli will reach these water bodies.

7. The long detention aerobic pond represents the more effective system in the extinction of the typhoid bacillus excreted by the typhoid case or carrier.

IX.2 Recommendations

As the oxidation pond and its public health aspects have not been investigated in proportion to its use, there remain many questions which avail themselves to further investigation. These include the following:

1. Detailed studies of the longevities of other pathogenic gastro-enteric bacteria in the oxidation pond.

2. The pathogenic viruses in the oxidation pond and their capability of being vector-borne by insects, birds and animals.

3. A thorough investigation into the ecology of the anaerobic pond and the effect that loading rates have upon it.

4. Studies related to the re-circulation of algae into the anaerobic pond.

5. The use of columns as applied to this investigation is recommended for future investigations of this nature.

It is suggested that future studies of oxidation ponds include the biological and biochemical aspects of the pond's operation. Investigations concerned with only the chemical aspects tend to be very limited.

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